

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:)	Group Art Unit: 1644
GELFAND et al.)	Examiner: Huynh, Phuong N.
Serial No.: 09/809,753)	Confirmation No.: 5001
Filed: March 14, 2001)	<u>SUGGESTION FOR INTERFERENCE</u>
Atty. File No.: 2879-74)	<u>UNDER 37 C.F.R. § 41.202</u>
For: "METHOD FOR REDUCING ALLERGEN-INDUCED AIRWAY HYPERRESPONSIVENESS")	<i>Submitted Via EFS-Web</i>

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

Pursuant to 37 C.F.R. § 41.202, Applicants submit this Suggestion for Interference and respectfully request that the Office declare an interference between the present application and U.S. Patent No. 6,743,429 to Cadieux ("the '429 patent"), which is assigned on its face to Sherbrooke University.

Applicants present below the information requested under 37 C.F.R. § 41.202 under headings corresponding to the subsections of § 41.202, in order to facilitate the Office's consideration of this information.

1. Identification of Patent With Which Applicant Seeks an Interference

Applicants seek an interference between the present U.S. Application No. 09/809,753 ("the '753 application"), filed on March 14, 2001, and claiming priority of U.S. Provisional Application No. 60/189,622. filed March 14, 2000, and U.S. Patent No. 6,743,429 ("the '429 patent"), filed on December 30, 1999, claiming a foreign priority date of December 24, 1999, and issued to Alain Cadieux on June 1, 2004.

2. Identification of Interfering Subject Matter

The interfering subject matter of this suggested interference is drawn generally to methods to inhibit allergen-induced airway hyperresponsiveness in a mammal. More specifically, claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application and claims 1-13 of the '429 patent define interfering subject matter. Section 4 below explains in detail how the claims from the '753 application and the '429 patent interfere and how they correspond to the proposed count.

3. Proposed Count

In accordance with 37 C.F.R. § 41.202(a)(2), Applicants propose the following count to define the interfering subject matter of this suggested interference based on independent claim 1 of the '753 application and dependent claim 3 of the '429 patent.

Proposed Count

A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.

The proposed count is identical to independent claim 1 of the '753 application, which is similar to dependent claim 3 of the '429 patent. The proposed count recites “[a] method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP); wherein said mammal has allergen-induced airway responsiveness.” Claim 3 of the '429 patent, which depends from claim 1, is directed to “[a] method of reducing a

stimulus-induced airway response selected from [several options including airway hyperreactivity],” wherein the stimulus is selected from an allergen and an agonist. In view of its dependency from claim 1, claim 3 further recites administering by inhalation to a subject at risk of experiencing the stimulus-induced airway response an agent selected from various specific types of CGRP.

Thus, both claim 3 of the ‘429 patent and the proposed count are directed to reducing or inhibiting allergen-induced airway hyperresponsiveness. Claim 3 of the ‘429 patent actually uses the equivalent term “airway hyperreactivity.” The ‘429 patent appears to use the terms “hyperreactivity” and “hyperresponsiveness” interchangeably. See the ‘429 patent, e.g., at column 8, lines 4 to 52. Moreover, the methods of both the proposed count and claim 3 of the ‘429 patent comprise administering CGRP to a mammal that has allergen-induced airway hyperresponsiveness or to a subject at risk of experiencing allergen-induced airway hyperresponsiveness.

The count should be limited to allergen-induced airway hyperresponsiveness in view of certain prior art that arose during the prosecution in the present application. Specifically, in the present application, Applicants amended claims directed generally to inhibition of airway hyperresponsiveness to recite the more specific inhibition of **allergen**-induced airway hyperresponsiveness to successfully remove rejections over 1996 prior art directed to hyperpnea-induced airway hyperresponsiveness. See Office Action mailed in this application on July 29, 2002, at pages 7 to 8, and at pages 10 to 16, in which the Examiner asserted prior art rejections involving Nagase et al., Am. J. Respir. Crit. Care, Med., 154:1551-56 (1996) (“Nagase”); the Amendment and Response filed January 29, 2003; the Amendment and Response filed February 12, 2004, and the

Office Action mailed April 21, 2004, in which the rejections over Nagase were no longer asserted.¹ Applicants successfully argued that hyperpnea-induced airway hyperresponsiveness is induced by a different stimulus than an allergen. Specifically, hyperpnea-induced airway hyperresponsiveness results from “deep and rapid breathing, particularly of cold, dry air such as occurs in exercise-induced asthma (Nagase et al., page 1551, first paragraph).” Amendment and Response filed February 12, 2004, at page 13.

In view of that prosecution history, the count should not recite inhibition of any stimulus-induced airway hyperresponsiveness, because such a generic method would encompass the 1996 prior art method directed to hyperpnea-induced airway hyperresponsiveness. See 37 C.F.R. § 41.208(c)(2) (“[a] party moving to add or amend a count must show the count is patentable over prior art”). Accordingly, the count should be limited to a method of inhibiting allergen-induced airway hyperresponsiveness as set forth in claim 1 of the present application.

Next, the proposed count recites “wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.” Claims 1 and 3 of the ‘429 patent do not recite that element. If a method involving administration of CGRP provides no inhibition or reduction of allergen-induced airway hyperresponsiveness in the subject as compared to in the absence of administration of CGRP, however, the method really would not be a method of inhibiting or reducing hyperresponsiveness by administering CGRP as required by claims 1 and 3 of the ‘429 patent. Thus, that element of the

¹ A copy of Nagase is attached as Appendix D.

proposed count should be part of any showing of a reduction to practice of an embodiment within the scope of claims 1 and 3 of the '429 patent.

Accordingly, applicants request that claim 1 of the '753 application be used as the count in the interference.

4. Claims Corresponding to the Count and Claim Chart Comparing Interfering

Subject Matter

A claim should be designated as corresponding to the count if, considering the count as prior art, the claim would be unpatentable over the count under 35 U.S.C. § 102 or § 103. 37 C.F.R. § 41.207(b)(2). In addition to the explanation below, Applicants also provide in Appendix A a claim chart comparing the count with at least one claim from each party as required under 37 C.F.R. § 41.202(a)(3). Furthermore, Appendix B provides a clean copy of claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application that contain interfering subject matter as defined in the count, and Appendix C provides a clean copy of claims 1-13 of the '429 patent that contain the interfering subject matter.

A. Claims of the '753 application

Claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application correspond to the proposed count. Independent claim 1 corresponds to the proposed count because the proposed count is identical to claim 1. Thus, claim 1 is anticipated by the proposed count under 35 U.S.C. § 102. Independent claim 46 and dependent claims 3-10, 12-14, 20-26, 29, 30, 43, and 44 of the '753 application would have been obvious over the proposed count under 35 U.S.C. § 103.

The proposed count and claim 1 of the '753 application recites:

A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.

Independent claim 46 is similar to the proposed count, but further recites a specific condition of the mammal. Specifically, claim 46 recites: “wherein said mammal has allergen-induced airway hyperresponsiveness in response to a concentration of methacholine that causes a 20% fall in FEV1 (PC20FEV1), wherein said concentration is less than the concentration required to cause a 20% fall in FEV1 (PC20FEV1) in non-allergen-sensitized mammals.” That definition of the mammal which distinguishes the mammal from non-allergen-sensitized mammals would not render claim 46 patentable over the proposed count.

Dependent claim 3 defines the mammal as having been sensitized to an allergen and having been exposed to, or at risk of being exposed to, an amount of the allergen that would induce airway hyperresponsiveness (AHR) in the absence of CGRP. That definition of the mammal would not render claim 3 patentable over the proposed count.

Dependent claims 4 to 10, 12 to 14, 21 to 24, and 29 recite particular dosing regimens, such as timing, amounts, and modes of delivery. Those dosing regimens would not render those claims patentable over the proposed count.

Dependent claims 25, 26, and 43 recite particular responses of the mammal, which would not render those claims patentable over the proposed count.

Dependent claim 20 recites that the CGRP targets particular cells in the lung, which would not render that claim patentable over the proposed count.

Dependent claim 30 defines the mammal as human, which would not render that claim patentable over the proposed count.

Dependent claim 44 recites that the CGRP is human α CGRP, which would not render that claim patentable over the proposed count.

B. Claims of the '429 patent

Claims 1-13 of the '429 patent correspond to the count. Claim 1 of the '429 patent recites:

A method of reducing a stimulus-induced airway response selected from the group consisting of airway constriction, bronchospasm, airway hyperreactivity, eosinophil accumulation in bronchial walls, an increase in airway resistance, or combinations thereof, said method comprising:

administering by inhalation to a subject at risk of experiencing said stimulus-induced airway response a therapeutically effective amount of an agent selected from the group consisting of: (a) human calcitonin gene-related peptide (human CGRP); (b) rat CGRP; (c) the diacetoamidomethyl cysteine form of (a); and (d) the diacetoamidomethyl cysteine form of (b);

wherein said agent is administered prior to said airway response and wherein said method results in no or substantially no haemodynamic side effects.

Claim 1 encompasses a method of reducing or inhibiting allergen-induced airway hyperresponsiveness according to the proposed count. In fact, dependent claim 3 explicitly recites that the stimulus can be an allergen. Also, the '429 patent appears to use the terms "hyperreactivity" and "hyperresponsiveness" interchangeably. See the '429 patent, e.g., at column 8, lines 4 to 52.

Claim 1 also encompasses such a method comprising administering CGRP to a mammal that has allergen-induced airway hyperresponsiveness according to the proposed count. Specifically, claim 1 recites that the method comprises “administering by inhalation to a subject at risk of experiencing said stimulus-induced airway response [including airway hyperresponsiveness as discussed above] a therapeutically effective amount of an agent selected from [particular types of CGRP].” The recitation in claim 1 that the administration is by inhalation does not distinguish claim 1 from the proposed count. Certainly, it would have been obvious to administer the CGRP by inhalation because the method is directed to reducing an airway response. It was certainly common to administer agents to reduce an airway response by inhalation. In fact, dependent claim 22 of the ‘753 application recites that the CGRP is administered by aerosol delivery. Also, the Examiner in the present application cited U.S. Patent No. 5,635,478, issued in June 1997, as showing administration of CGRP “into the respiratory tract such as the lungs by aerosol spray (See column 6, lines 34-35, in particular)”² See Office “Action mailed in the present application on July 29, 2002, at page 9. The cited section of U.S. Patent No. 5,635,478 reads: “The compositions of this invention [including CGRP] can also be in the form of an aerosol for inhalation” See U.S. Patent No. 5,635,478 at column 6, lines 34 to 35.

Finally, claim 1 recites “wherein said agent is administered prior to said airway response and wherein said method results in no or substantially no haemodynamic side effects.” First, it would have been obvious to administer the agent prior to said airway response. In fact, the Examiner in the present application cited Nagase (Appendix D)

² A copy of U.S. Patent No. 5,635,478 is attached as Appendix E.

as teaching administering CGRP “two minutes . . . prior to an AHR provoking stimulus such as hyperpnea challenge or methacholine” See Office Action mailed in the present application on July 29, 2002, at page 7. As discussed above, Nagase was published in 1996.

Second, the ‘429 patent states that “[o]ther advantages attributable to CGRP are that it does not cause undesirable haemodynamic side effects when administered by inhalation administration” See ‘429 patent at column 4, lines 41 to 44. Accordingly, that claimed element would have been inherent to administration of CGRP by inhalation. As discussed above, administration of CGRP by inhalation would have been obvious.

Thus, the recitation in claim 1 “wherein said agent is administered prior to said airway response and wherein said method results in no or substantially no haemodynamic side effects,” would not have distinguished claim 1 from the proposed count. Accordingly, claim 1 of the ‘429 patent would have been obvious in view of the proposed count, and thus, corresponds to the proposed count.

Claim 2 of the ‘429 patent recites: “The method of claim 1 wherein said stimulus is selected from the group consisting of a non-specific stimulus and exposure to an irritant.” Claim 3 of the ‘429 patent recites: “The method of claim 2 wherein said irritant is selected from the group consisting of an allergen and an agonist.”

The analysis of claim 1 of the ‘429 patent above already considered the option of the stimulus being an allergen. Thus, claim 3, which recites that the stimulus is an allergen would have been unpatentable in view of the proposed count. Also, claim 3 indicates that an allergen is encompassed within the term “irritant” in claim 2.

Accordingly, claim 2, which recites that the stimulus is an irritant would have been unpatentable in view of the proposed count.

When suggesting an interference, the rules state that the applicant should show why the claims interfere within the meaning of 37 C.F.R. § 41.203(a). See 37 C.F.R. § 41.202(a)(3). An interference is appropriate between an application and an unexpired patent of different parties if the subject matter claimed by one party would have, if prior art, anticipated or rendered obvious the subject matter of a claim of the opposing party and vice versa. 37 C.F.R. § 41.203(a). That is, an interference is appropriate when the application and patent contain claims to the same patentable invention. The Board uses a two-way test to determine the presence of interfering subject matter. See *Eli Lilly & Co. v. Board of Regents of the University of Washington*, 334 F.3d 1264, 67 U.S.P.Q.2d 1161 (Fed. Cir. 2003).

In the present case, the Examiner has already asserted that the '429 patent claims the same patentable invention as claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application. See Office Action mailed May 5, 2006, at page 3. Applying the two-way test to claim 1 of the '753 application and claim 3 of the '429 patent confirms the Examiner's conclusion. First, the analysis above establishes that claim 3 of the '429 patent would have been obvious in view of the proposed count. The proposed count is identical to claim 1 of the '753 application. Thus, the analysis above satisfies one direction of the two-way test.

The second part of the two-way test requires an analysis of whether claim 1 of the '753 application would have been anticipated or obvious in view of claim 3 of the '429 patent. As discussed above, claim 3 of the '429 patent is directed to a method of

reducing a stimulus-induced airway response, including the options of an allergen as the stimulus and airway hyperreactivity or hyperresponsiveness as the airway response. The method of claim 3 comprises administering by inhalation to a subject at risk of experiencing said stimulus-induced airway response a therapeutically effective amount of an agent selected from particular types of CGRP. Accordingly, claim 3 of the '429 patent would have shown "[a] method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP), wherein said mammal has allergen-induced airway hyperresponsiveness" as recited in claim 1 of the '753 application.

Claim 1 of the '753 application further recites "wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP." As noted above, if a method involving administration of CGRP provides no inhibition or reduction of allergen-induced airway hyperresponsiveness in the subject as compared to in the absence of administration of CGRP, the method would not be a method of inhibiting or reducing hyperresponsiveness by administering CGRP as set forth in claim 3 of the '429 patent. Thus, that element of claim 1 of the '753 application should be understood from claim 3 of the '429 patent.

Accordingly, claim 1 of the '753 application would not be patentable over claim 3 of the '429 patent. Thus, claim 1 of the '753 application and claim 3 of the '429 patent claim the same patentable invention within the meaning of 37 C.F.R. § 41.203(a) as shown by this analysis under the two-way test.

Dependent claim 4 of the '429 patent recites that the airway response is selected from the early or late phase responses induced by the stimulus. The '429 patent does not indicate that there is any phase of the response other than a late or early phase response. Accordingly, it would appear that any method of inhibiting an allergen-induced airway hyperresponsiveness would impact either the early or late phase of the response. Thus, the proposed count would render unpatentable claim 4 of the '429 patent.

Dependent claim 5 of the '429 patent recites that the airway comprises lower segments of the tracheobronchial tree. The '429 patent attributes the reduction of the airway response to the administration of CGRP. There is no indication that anything other than administration of CGRP resulted in such reduction in the airway response in the lower segments of the tracheobronchial tree. See '429 patent, e.g., at column 13, lines 47 to 34. Accordingly, that claimed element would have been inherent to administration of CGRP, which clearly is shown by the proposed count. Thus, the proposed count would render unpatentable claim 5 of the '429 patent.

Dependent claims 6 to 9 of the '429 patent are directed to certain particular subsets of the different CGRP's set forth in claim 1 of the '429 patent. The proposed count is directed to administration of CGRP. There is no indication that use of any particular species of CGRP would be patentable over the genus of CGRP's according to the proposed count. Thus, the proposed count would render unpatentable claims 6 to 9 of the '429 patent.

Dependent claim 10 of the '429 patent recites that the agent is administered such that it contacts the respiratory epithelium of the subject. The '429 patent does not

indicate that anything other than administration of CGRP by inhalation would result in the CGRP contacting the respiratory epithelium. Accordingly, that claimed element would have been inherent to administration of CGRP by inhalation. As discussed above, administration of CGRP by inhalation would have been obvious. Moreover, the face of the '429 patent includes the citation: "Tschirhart et al.; "Evidence for the Involvement of Calcitonin Gene-related Peptide in the Epithelium-dependent Contraction of Guinea-pig Trachea in Response to Capsaicin"; Naunyn-Schmied. Arch. Pharmacol; vol. 342; 1990; pp. 177-181. Accordingly, it is apparent that the prior art discussed the effect of CGRP on epithelium-dependent contraction in trachea. Thus, the proposed count would render unpatentable claim 10 of the '429 patent.

Dependent claim 11 of the '429 patent recites that the agent has a purity of at least about 95 to 98%. Because the CGRP in the proposed count is being administered to a mammal, including a human, it would have been obvious to administer CGRP having a purity of at least about 95 to 98%. Thus, the proposed count would render unpatentable claim 11 of the '429 patent.

Dependent claim 12 of the '429 patent recites that the agent is dispersed within a composition comprising a pharmaceutically acceptable excipient, and/or a liquid or solid carrier. Because the CGRP in the proposed count is being administered to a mammal, it would have been obvious to administer CGRP dispersed within a composition comprising a pharmaceutically acceptable excipient, and/or a liquid or solid carrier. In fact, U.S. Patent No. 5,635,478 (Appendix E), issued in June 1997, which was cited by the Examiner in the prosecution of the present application, discusses various

pharmaceutical formulations of CGRP at column 5, line 52, to column 6, line 59. Thus, the proposed count would render unpatentable claim 12 of the '429 patent.

Dependent claim 13 of the '429 patent depends from claim 12 and recites that the composition is formulated as an aerosol or dry powder. As discussed above, the Examiner in the present application cited U.S. Patent No. 5,635,478 (Appendix E), issued in June 1997, as showing administration of CGRP "into the respiratory tract such as the lungs by aerosol spray (See column 6, lines 35, in particular)" See Office "Action mailed in the present application on July 29, 2002, at page 9. The cited section of U.S. Patent No. 5,635,478 reads: "The compositions of this invention [including CGRP] can also be in the form of an aerosol for inhalation" See U.S. Patent No. 5,635,478 at column 6, lines 34 to 35. Thus, the proposed count would render unpatentable claim 13 of the '429 patent.

Accordingly, claims 1-13 of the '429 patent would have been obvious in view of, and therefore, correspond to, the proposed count.

5. Reasons Why Applicants Will Prevail on Priority

As required under 37 C.F.R. §§ 41.202(a)(4) and 41.202(d), Applicants submit that Applicants will prevail on priority. The earliest constructive reduction to practice for the '753 application is March 14, 2000, which is the filing date of U.S. Provisional Application No. 60/189,622. The apparent earliest constructive reduction to practice for the '429 patent is December 24, 1999, which is the filing date of Canadian Application No. 2292902. Applicants use the language "apparent earliest constructive reduction" in this paper because that is the language used in Rule 41.202(d)(1). Applicants, however, do not concede that the '492 patent is entitled to priority benefit of that date.

Accordingly, the earliest constructive reduction to practice for the '753 application is later than the apparent earliest constructive reduction practice for the '429 patent. Thus, Applicants submit the following showing under 37 C.F.R. § 41.202(d)(1). Under 37 C.F.R. § 41.202(e)(4), this showing of priority is sufficient, because it would, if unrebutted, support a determination of priority in favor of Applicants.

The following showing establishes that Applicants actually reduced to practice an embodiment within the scope of the proposed count at least by August 26, 1999, which is before the apparent earliest constructive reduction to practice (December 24, 1999) for the '429 patent. In the event, Applicants get into a priority contest with the '429 patent or another party, they in no way are limited by the August 26, 1999, date or the particular proofs provided here. Applicants reserve their right to rely upon different proofs and different dates.

"Proof of actual reduction to practice requires satisfaction of a two pronged test: (1) the party must have constructed an embodiment that met every element of the interference count, and (2) the embodiment must have operated for its intended purpose." *Eaton v. Evans*, 204 F.3d 1094, 1097, 53 U.S.P.Q.2d 1696, 1698 (Fed. Cir. 2000). The Declarations of Dr. Azzeddine Dakhama and Dr. Arihiko Kanehiro, including the attached exhibits, establish that Applicants actually reduced to practice an embodiment that met every element of the interference count by at least August 26, 1999. Those Declarations are attached as Appendices F and G. Dr. Dakhama is a co-inventor of the '753 application. Dr. Kanehiro was not a co-inventor and he provides corroboration of the actual reduction to practice by at least August 26, 1999.

As discussed above, the proposed count reads as follows:

A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.

In summary, both Declarations discuss records of an experiment that was conducted by Dr. Dakhama and Dr. Kanehiro. Both Dr. Dakhama and Dr. Kanehiro stated that, on August 26, 1999, they recognized that the data from that experiment demonstrated that administration of CGRP to a mouse that has allergen-induced airway hyperresponsiveness (AHR) inhibited allergen-induced AHR in the mouse as compared to in the absence of administration of CGRP. See Dr. Dakhama's Declaration ("Dakhama Declaration") at pages 12 to 13, paragraph 24;, and Dr. Kanehiro's Declaration ("Kanehiro Declaration") at pages 12 to 13, paragraph 24. Accordingly, those Declarations establish that Applicants had actually reduced to practice an embodiment that met every limitation of the proposed count and showed that the embodiment operated for its intended purpose.

The Declarations are now discussed in more detail. Dr. Dakhama confirms that he is a co-inventor of the '753 application. Dakhama Declaration at page 1, paragraph 2. Dr. Kanehiro confirms that he is not an inventor of the subject matter of the proposed count. Kanehiro Declaration at page 2, paragraph 2.

Dr. Dakhama authenticates pages 1 and 2 of Exhibit A of both Declarations as being a printout from a computer entry that he entered into a computer as a record of the experiment. Dakhama Declaration at pages 1 to 2, paragraph 4. Dr. Dakhama also

authenticates page 3 of Exhibit A of both Declarations as an image of the computer information for that file which shows that the file was created on August 25, 1999, and it was last modified on August 27, 1999. *Id.* Dr. Dakhama states that he labeled the experiment described on pages 1 and 2 of Exhibit A of both Declarations “Experiment 23JL99,” which meant that he initiated the experiment on July 23, 1999. Dr. Kanehiro authenticates pages 4 to 7 of Exhibit A of both Declarations as photocopies of his handwritten notebook pages, which include raw data that he recorded for the experiment on August 25 and 26, 1999. Kanehiro Declaration at pages 3 and 4, paragraph 5.

Both Dr. Dakhama and Dr. Kanehiro explain in detail that Dr. Kanehiro’s handwritten pages (pages 4 to 7 of Exhibit A) describe the same experiment discussed in the printout of Dr. Dakhama’s entry into the computer (pages 1 and 2 of Exhibit A). Dakhama Declaration at pages 2 to 3, paragraph 5. Kanehiro Declaration at pages 3 to 4, paragraph 5. Both Dr. Dakhama and Dr. Kanehiro explain that Dr. Dakhama performed an earlier part of the experiment, and Dr. Kanehiro performed a later part of the experiment in Dr. Dakhama’s presence. Dakhama Declaration at page 1, paragraph 3. Kanehiro Declaration at page 2, paragraph 3. Dr. Kanehiro recorded the data he obtained in the experiment as shown at pages 4 to 7 of Exhibit A, and Dr. Dakhama typed that same data into the computer as shown on pages 1 to 2 of Exhibit A. Dakhama Declaration at pages 2 to 3, paragraph 5. Kanehiro Declaration at pages 3 to 4 paragraph 5. Thus, completion of the experiment by Dr. Dakhama and Dr. Kanehiro on August 26, 1999, is corroborated.

Both Dr. Dakhama and Dr. Kanehiro state that they were skilled in the art in the field of this experiment. Dakhama Declaration at page 3, paragraph 6. Kanehiro Declaration at page 4, paragraph 6. Both Dr. Dakhama and Dr. Kanehiro state that page 1 of Exhibit A shows that “the purpose of the experiment was to study whether calcitonin gene related peptide (CGRP) would inhibit methacholine (MCh) induced airway hyperresponsiveness (AHR) in mice sensitized and exposed to the allergen ovalbumin (OVA), which was a mouse model of asthma.” *Id.*

Both Dr. Dakhama and Dr. Kanehiro state that this mouse model “was known by those skilled in this field as an acceptable model for testing allergen-induced AHR.” Dakhama Declaration at pages 3 to 4, paragraph 7. Kanehiro Declaration at pages 4 to 5, paragraph 7. Both then cite exemplary 1997 articles describing the mouse model and then explain that those articles “provide experimental data showing that the model is a valid model for allergen-induced AHR.” *Id.* Thus, the Declarations establish that the experiment they performed was regarded in the art as an acceptable model to test for allergen-induced AHR as set forth in the proposed count.

Dr. Dakhama and Dr. Kanehiro then describe the experiment in detail. Dakhama Declaration at pages 4 to 12, paragraphs 8 to 24. Kanehiro Declaration at pages 5 to 13, paragraphs 8 to 24. Paragraphs 8 to 10 describe how mice were sensitized to the allergen ovalbumin (OVA), then challenged with OVA, and then subjected to increasing doses of methacholine (MCh). Citing Takeda et al., paragraph 10 states that “[i]t was known that in such a model, after exposure to the provoking agent MCh, mice that have been sensitized to OVA and subsequently challenged with OVA show increased AHR

compared to control mice that have not been both sensitized and challenged with the allergen OVA. Thus, it is clear that in the model the AHR is allergen-induced.”

Paragraphs 11 to 12 then describe the method used by Dr. Kanehiro in the experiment to measure two values: lung resistance (R_L) and dynamic compliance (C_{dyn}). Paragraph 13 discusses the effects on the mice that are reflected by the values. Citing Takeda et al., paragraph 13 then states that “[i]ncreases in R_L and decreases in C_{dyn} correlate with increased AHR.”

Paragraphs 14 to 24 then discuss the actual experiment, which tested whether administration of CGRP inhibited allergen-induced AHR. Both Dr. Dakhama and Dr. Kanehiro state that the experiment they conducted was an accepted method of testing whether a test agent inhibited allergen-induced AHR in mammals compared to mammals that did not receive treatment with the test agent. Dakhama Declaration at page 7, paragraph 15. Kanehiro Declaration at page 8, paragraph 15.

The Declarations discuss Dr. Dakhama’s preparation of four groups of mice for the experiment. The first group of mice were mice that had allergen-induced airway hyperresponsiveness in view of their sensitization to, and challenge with, OVA. Both Declarations describe the first group of mice (Dakhama Declaration at page 8, paragraph 17, Kanehiro Declaration at pages 8 to 9, paragraph 17) and confirm that such mice have allergen-induced AHR (Dakhama Declaration at page 5, paragraph 10, Kanehiro Declaration at pages 5 to 6, paragraph 10). The first group of mice also were administered CGRP. Dakhama Declaration at page 8, paragraph 17. Kanehiro Declaration at pages 8 to 9, paragraph 17.

The second group of mice also were mice that had allergen-induced airway hyperresponsiveness in view of their sensitization to, and challenge with, OVA. Both Declarations describe the second group of mice (Dakhama Declaration at pages 8 to 9, paragraph 18, Kanehiro Declaration at page 9, paragraph 18) and confirm that such mice have allergen-induced AHR (Dakhama Declaration at page 5, paragraph 10, Kanehiro Declaration at pages 5 to 6, paragraph 10). The second group of mice also were administered CGRP and CGRP antagonist. Dakhama Declaration at pages 8 to 9, paragraph 18. Kanehiro Declaration at page 9, paragraph 18.

The third group of mice were negative control mice, which were not sensitized and not challenged with OVA, and which also were not administered CGRP. Dakhama Declaration at page 9, paragraph 19. Kanehiro Declaration at pages 9 to 10, paragraph 19.

The fourth group of mice were mice that had allergen-induced airway hyperresponsiveness in view of their sensitization to, and challenge with, OVA. Both Declarations describe the fourth group of mice (Dakhama Declaration at pages 9 to 10, paragraph 20, Kanehiro Declaration at page 10, paragraph 20) and confirm that such mice have allergen-induced AHR (Dakhama Declaration at page 5, paragraph 10, Kanehiro Declaration at pages 5 to 6, paragraph 10). The fourth group of mice were positive control mice because they were not administered CGRP. Dakhama Declaration at pages 9 to 10, paragraph 20. Kanehiro Declaration at page 10, paragraph 20.

Dr. Dakhama provided the mice from those four groups to Dr. Kanehiro so that he could expose the mice to the MCh and make the measurements of lung resistance

(R_L) and dynamic compliance (C_{dyn}). Dakhama Declaration at page 10, paragraph 21. Kanehiro Declaration at pages 10 to 11, paragraph 21. The recordation of those values by Dr. Kanehiro is discussed in both Declarations at pages 10 to 11, paragraphs 21 and 22.

Dr. Dakhama and Dr. Kanehiro discuss the evaluation of the obtained data at pages 11-13, paragraphs 23 and 24, of their Declarations. Specifically, both state that:

[t]hese data were evaluated by comparing the changes in R_L in response to increasing doses of MCh, represented by the first number recorded for each mouse, in the presence and absence of CGRP. Changes in C_{dyn} were also evaluated by comparing the change in this number in response to increasing doses of MCh, represented by the second number recorded for each mouse, in the presence and absence of CGRP.

Dakhama Declaration at pages 11 to 12, paragraph 23. Kanehiro Declaration at pages 11 to 12, paragraph 23.

Both further state that:

Visual inspection of the raw data showed that if the positive and negative control mice are compared, the R_L values for the positive control mice appeared to be significantly higher in response to increasing doses of MCh as compared to the R_L values for the negative control mice. Compare, for example, the R_L values for mouse 1 in Group 3 (mouse 1 in the 8/26 data) to mouse 1 in Group 4 (mouse 4 in the 8/26 data). With respect to C_{dyn} , C_{dyn} values for the positive control mice appeared to be significantly lower in response to increasing doses of MCh as compared to the C_{dyn} values for the negative control mice, which was expected.

Dakhama Declaration at pages 11 to 12, paragraph 23. Kanehiro Declaration at pages 11 to 12, paragraph 23. Thus, the significantly higher R_L values and the significantly lower C_{dyn} values for the positive control mice compared to those values for the negative control mice confirmed an allergen-induced AHR in the positive control mice as explained by both Dr. Dakhama and Dr. Kanehiro (Dakhama Declaration at page 5,

paragraph 10, and at pages 6 to 7, paragraph 13, Kanehiro Declaration at pages 5-6, paragraph 10, and at page 7, paragraph 13).

Both Dr. Dakhama and Dr. Kanehiro then state that the R_L and C_{dyn} values for the mice treated with CGRP “appeared to be more similar to negative control mice [which did not have allergen-induced AHR] than the positive control mice [which had allergen-induced AHR and which were not administered CGRP]. . . .” Dakhama Declaration at page 12, paragraph 23. Kanehiro Declaration at page 12, paragraph 23.

Both then state that “[i]n contrast, viewing, for example, the R_L values for mouse 5 of Group 2 (mouse 5 in the 8/25 data), which represented CGRP antagonist mice, these values appeared to be more similar to the positive control than the negative control mice, as did the C_{dyn} values, indicating that the inhibitory effects of CGRP were abolished in the presence of a CGRP antagonist.” *Id.*

Both then stated that “[t]herefore, I concluded from looking at the raw data for this experiment that CGRP inhibited AHR in allergen-sensitized and challenged mice as compared to in the absence of CGRP, and furthermore, that the effects were directly due to the CGRP, since the CGRP antagonist reversed that result. *Id.*

Both Dr. Dakhama and Dr. Kanehiro then state that:

I confirm that the experiment described in Exhibit A was completed on August 26, 1999. I also confirm that on that day, I recognized that the data showed that the administration of CGRP to allergen-sensitized and challenged mice inhibited allergen-induced airway hyperresponsiveness (AHR) in the mice, as compared to in the absence of administration of CGRP. I also confirm that on that day, I recognized that the data from that experiment demonstrated that administration of CGRP to a mouse that has allergen-induced AHR inhibited allergen-induced AHR in the mouse as compared to in the absence of administration of CGRP. It is my opinion that anyone skilled in the relevant field reading these data would have arrived at the same conclusion on August 26, 1999.

Dakhama Declaration at pages 12 to 13, paragraph 24. Kanehiro Declaration at pages 12 to 13, paragraph 24.

Thus, it is clear that at least by August 26, 1999, the experiment conducted by Dr. Kanehiro and Dr. Dakhama was an actual reduction to practice of an embodiment that met every limitation of the proposed count. Also, it is clear that at least by August 26, 1999, they both recognized that they had reduced to practice that embodiment. Specifically, the valid art recognized experiment was:

A method to inhibit allergen-induced airway hyperresponsiveness in a mammal [specifically a mouse], comprising administering to a mammal [specifically a mouse] a calcitonin gene related peptide (CGRP);

wherein said mammal [specifically a mouse] has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal [specifically a mouse] as compared to in the absence of administration of said CGRP.

Moreover, in view of the positive results the embodiment clearly operated for its intended purpose. Also, Dr. Kanehiro's involvement and recordation of data from the experiment provides corroboration of the actual reduction to practice by a noninventor. This showing of priority, if unrebutted, supports a determination of priority in favor of Applicants in view of an actual reduction to practice by at least August 26, 1999, which is before the December 24, 1999, apparent earliest constructive reduction to practice for the '429 patent.

Thus, this showing of priority is sufficient to show why Applicants would prevail on priority under 37 C.F.R. §§ 41.202(a)(4), (d)(1), and (e)(1).

6. Claims Added or Amended to Provoke Interference

Rule 41.202(a)(5) requires a claim chart showing the location of written description support within the specification for claims that have been added or amended to provoke an interference. All of the claims of the '753 application were already pending when the Examiner first asserted the rejection in view of the '429 patent in the Office Action mailed August 10, 2005. Thus, no claims were added or amended to provoke an interference. Accordingly, Applicants do not believe that a claim chart is required under Rule 41.202(a)(5).

7. Constructive Reduction to Practice of the Proposed Counts

37 C.F.R. § 41.202(a)(6) requires a chart showing where the disclosure provides a constructive reduction to practice within the scope of the proposed count for each constructive reduction to practice for which the applicant wishes to be accorded benefit. The chart in Appendix H shows that U.S. Provisional Application No. 60/189,622, filed March 14, 2000, provides a constructive reduction to practice of an embodiment within the scope of the proposed count. The chart in Appendix I shows that the '753 application, filed March 14, 2001, provides a constructive reduction to practice of an embodiment within the scope of the count. Accordingly, Applicants should be accorded benefit of U.S. Provisional Application No. 60/189,622, filed March 14, 2000, and the '753 application, filed March 14, 2001.

8. Conclusion

Applicants respectfully request that an interference be declared using the proposed count set forth in Section 3 of this paper, with claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application and claims 1-13 of the '429 patent corresponding to the count. Applicants additionally request that Applicants be accorded benefit of the filing dates of U.S. Provisional Application No. 60/189,622, filed March 14, 2000, and the '753 application, filed March 14, 2001,

Please grant any extensions of time required to enter this Suggestion for Interference and charge any additional required fees to Deposit Account No. 19-1970.

Respectfully submitted,

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Date: February 25, 2008

APPENDIX A

Claim chart comparing at least one claim from each party to the proposed count

Count 1	Claims of the '753 application	Claims of the '492 patent
<p>A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);</p> <p>wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.</p>	<p>Claim 1</p> <p>A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);</p> <p>wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.</p>	<p>Claim 1</p> <p>A method of reducing a stimulus-induced airway response selected from the group consisting of airway constriction, bronchospasm, airway hyperreactivity, eosinophil accumulation in bronchial walls, an increase in airway resistance, or combinations thereof, said method comprising:</p> <p>administering by inhalation to a subject at risk of experiencing said stimulus-induced airway response a therapeutically effective amount of an agent selected from the group consisting of: (a) human calcitonin gene-related peptide (human CGRP); (b) rat CGRP; (c) the diacetoamidomethyl cysteine form of (a); and (d) the diacetoamidomethyl cysteine form of (b);</p> <p>wherein said agent is administered prior to said airway response and wherein said method results in no or substantially no haemodynamic side effects.</p> <p>Claim 2</p> <p>The method of claim 1 wherein said stimulus is selected from the group consisting of a non-specific stimulus and exposure to an irritant.</p> <p>Claim 3</p> <p>The method of claim 2 wherein said irritant is selected from the group consisting of an allergen and an agonist.</p>

Appendix B

Claims 1, 3-10, 12-14, 20-26, 29, 30, 43, 44, and 46 of the '753 application containing interfering subject matter as defined in the count

1. A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.

3. The method of Claim 1, wherein said mammal has been sensitized to an allergen and has been exposed to, or is at risk of being exposed to, an amount of said allergen that is sufficient to induce airway hyperresponsiveness (AHR) in said mammal in the absence of said CGRP.

4. The method of Claim 1, wherein said method further comprises monitoring said mammal to detect whether AHR in said mammal is inhibited, wherein if AHR is detected in said mammal, additional amounts of said CGRP are administered until AHR is not detected in said mammal.

5. The method of Claim 1, wherein said CGRP is administered within a time period of between 48 hours or less prior to exposure to an AHR provoking stimulus that is sufficient to induce AHR, and within 48 hours or less after the detection of the first symptoms of AHR.

6. The method of Claim 1, wherein said CGRP is administered upon the detection of the first symptoms of AHR.

7. The method of Claim 1, wherein said CGRP is administered within 1 hour after the detection of the first symptoms of AHR.

Appendix B

8. The method of Claim 1, wherein said CGRP is administered within 12 hours or less prior to exposure to a AHR provoking stimulus that is sufficient to induce AHR.

9. The method of Claim 1, wherein said CGRP is administered within 2 hours or less prior to exposure to an AHR provoking stimulus that is sufficient to induce AHR.

10. The method of Claim 1, wherein said CGRP is administered to said mammal every one to two days.

12. The method of Claim 1, wherein said CGRP is administered at a dose of from about $0.1 \mu\text{g} \times \text{kilogram}^{-1}$ and about $20 \mu\text{g} \times \text{kilogram}^{-1}$ body weight of said mammal.

13. The method of Claim 1, wherein said CGRP is administered at a dose of from about $0.1 \mu\text{g} \times \text{kilogram}^{-1}$ and about $10 \mu\text{g} \times \text{kilogram}^{-1}$ body weight of said mammal.

14. The method of Claim 1, wherein said CGRP is administered at a dose of from about $0.1 \mu\text{g} \times \text{kilogram}^{-1}$ and about $5 \mu\text{g} \times \text{kilogram}^{-1}$ body weight of said mammal.

20. The method of Claim 1, wherein said CGRP is targeted to cells in the lung of said mammal selected from the group consisting of smooth muscle cells and epithelial cells.

21. The method of Claim 1, wherein said CGRP is administered by direct delivery of said CGRP to the lung of said mammal.

22. The method of Claim 1, wherein said CGRP is administered by aerosol delivery.

23. The method of Claim 1, wherein said CGRP is administered by parenteral delivery.

24. The method of Claim 1, wherein said CGRP is administered by oral delivery.

25. The method of Claim 1, wherein administration of said CGRP reduces the airway hyperresponsiveness of said mammal such that the FEV1 value of said mammal is improved by at least about 5%.

Appendix B

26. The method of Claim 1, wherein administration of said CGRP prevents airway hyperresponsiveness in said mammal when administered prior to exposure of said mammal to an AHR provoking stimulus that is sufficient to induce AHR.

29. The method of Claim 1, wherein said CGRP is administered in a pharmaceutically acceptable excipient.

30. The method of Claim 1, wherein said mammal is a human.

43. The method of Claim 1, wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal with statistical significance ($p < 0.05$) as compared to in the absence of administration of said CGRP.

44. The method of Claim 1, wherein the CGRP is human aCGRP.

46. A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness in response to a concentration of methacholine that causes a 20% fall in FEV₁ (PC₂₀FEV₁), wherein said concentration is less than the concentration required to cause a 20% fall in FEV₁ (PC₂₀FEV₁) in non-allergen-sensitized mammals; and

wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness induced by said concentration of methacholine in said mammal as compared to in the absence of administration of said CGRP.

Appendix C

Claims 1-13 of the '429 patent containing interfering subject matter as defined in the count

1. A method of reducing a stimulus-induced airway response selected from the group consisting of airway constriction, bronchospasm, airway hyperreactivity, eosinophil accumulation in bronchial walls, an increase in airway resistance, or combinations thereof, said method comprising:

administering by inhalation to a subject at risk of experiencing said stimulus-induced airway response a therapeutically effective amount of an agent selected from the group consisting of:

(a) human calcitonin gene-related peptide (human CGRP);

(b) rat CGRP;

(c) the diacetoamidomethyl cysteine form of (a); and

(d) the diacetoamidomethyl cysteine form of (b); wherein said agent is administered prior to said airway response and wherein said method results in no or substantially no haemodynamic side effects.

2. The method of claim 1 wherein said stimulus is selected from the group consisting of a non-specific stimulus and exposure to an irritant.

3. The method of claim 2 wherein said irritant is selected from the group consisting of an allergen and an agonist.

4. The method of claim 1 wherein said airway response is selected from the group consisting of early or late phase responses induced by said stimulus.

5. The method of claim 1 wherein said airway comprises the lower segments of the tracheobronchial tree.

Appendix C

6. The method of claim 1 wherein said agent is selected from the group consisting of human CGRP and rat CGRP.

7. The method of claim 1, wherein said agent is selected from the group consisting of the diacetoamidomethyl cysteine form of human CGRP and the diacetoamidomethyl cysteine form of rat CGRP.

8. The method of claim 1 wherein said agent is selected from the group consisting of human α CGRP and rat α CGRP.

9. The method of claim 1, wherein said agent is selected from the group consisting of the diacetoamidomethyl cysteine form of human α CGRP and the diacetoamidomethyl cysteine form of rat α CGRP.

10. The method of claim 1, wherein said agent is administered such that it contacts the respiratory epithelium of said subject.

11. The method of claim 1, wherein said agent has a purity of at least about 95 to 98%.

12. The method of claim 1, wherein said agent is dispersed within a composition comprising a pharmaceutically acceptable excipient, and/or a liquid or solid carrier.

13. The method of claim 12, wherein said composition is formulated as an aerosol or dry powder.

Appendix D

Nagase et al., Am. J. Respir. Crit. Care, Med., 154:1551-56 (1996)

Roles of Calcitonin Gene-Related Peptide (CGRP) in Hyperpnea-induced Constriction in Guinea Pigs

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It has been reported that hyperpnea-induced bronchoconstriction in guinea pigs is a potential model for exercise-induced asthma in humans. We hypothesized that calcitonin gene-related peptide (CGRP) could modulate leukotriene D₄ (LTD₄)-induced responses and be involved in the pathophysiology in this asthma model. We measured tracheal (Ptr) and alveolar pressure (PA) using alveolar capsules in open-chested, mechanically ventilated ($f = 1$ Hz, $V_T = 9$ ml/kg, PEEP = 4 cm H₂O) guinea pigs. Animals were intravenously pretreated with saline (SAL), CGRP(8-37) (CGRP receptor antagonist), CGRP, MK-571 (LTD₄ receptor antagonist), MK-886 (5-lipoxygenase inhibitor), or CGRP(8-37) + MK-571, and then underwent dry gas hyperpnea challenge (HC, 95% O₂-5% CO₂, 150 breaths/min, 7 min). We then calculated resistance of lung (R_L), tissue (R_{ti}), and airway (Raw). HC increased R_L, R_{ti}, and Raw in SAL controls (322 ± 27 , 430 ± 59 , $299 \pm 23\%$ baseline, respectively). MK-571, MK-886, and CGRP significantly reduced the responses to HC, while CGRP(8-37) enhanced HC-induced responses. Pretreatment with CGRP(8-37) and MK-571 in combination attenuated HC-induced constriction. In addition, pretreatment with CGRP reduced responses induced by intravenous administration of LTD₄. These observations suggest that CGRP might be involved in the pathophysiology of hyperpnea-induced constriction in guinea pigs via modulation of LTD₄-elicited responses. Nagase T, Ohga E, Katayama H, Sudo E, Aoki T, Matsuse T, Ouchi Y, Fukuchi Y. Roles of calcitonin gene-related peptide (CGRP) in hyperpnea-induced constriction in guinea pigs.

AM J RESPIR CRIT CARE MED 1996;154:1551-1556.

Hyperpnea-induced bronchoconstriction in guinea pigs has recently been proposed as an appropriate animal model of exercise-induced asthma in humans (1). This model shares many common features with human exercise-induced asthma in terms of the course of onset of constriction, spontaneous resolution, diminution of response with humidification of inspired gas, reproducibility on consecutive identical challenge, and the relationships between the amount of hyperpnea and the degree of response elicited (1-5). In this model, tachykinins released from airway C-fiber neurons are the central constrictive mediators (6), while bronchoactive eicosanoid mediators also participate in the pathogenesis of hyperpnea-induced bronchoconstriction (7).

Calcitonin gene-related peptide (CGRP) is another neuropeptide that is synthesized by sensory C fibers. CGRP is found in neuroepithelial cells of the lung and coexists with tachykinins in many airway sensory nerves (8). It has recently been shown that CGRP inhibits leukotriene release from platelet-activating factor-stimulated rat lung and ionophore-stimulated guinea pigs (9). In addition, Raud and colleagues (10) have demonstrated that

CGRP, but not substance P, is found to inhibit edema-promoting actions of inflammatory mediators in several species, indicating that sensory nerves may play an anti-inflammatory role. Whereas roles of tachykinins have been established in this model of hyperpnea-induced bronchoconstriction (6), little research has been performed to determine the effects of CGRP.

In the present study, we hypothesized that CGRP could modulate leukotriene D₄ (LTD₄)-induced responses and might be involved in the pathophysiology in this asthma model. To test this hypothesis, we studied the effects of CGRP(8-37) (a CGRP receptor antagonist) (11), CGRP, MK-571 (a LTD₄ receptor antagonist) (12), MK-886 (a 5-lipoxygenase inhibitor) (13), and pretreatment with CGRP(8-37) and MK-571 in combination on hyperpnea-induced bronchoconstriction.

In addition, it has recently been shown in several species including guinea pigs that both airway resistance and tissue resistance increase after exogenous constriction (14-20). Based on the recent observations that the different pharmacologic mechanisms including LTD₄-induced agonism are involved in the airway and lung tissue responses in the animal model of extrinsic asthma (21), it is expected that CGRP or LTD₄ antagonists might have differing effects on these compartments during hyperpnea-induced constriction. To elucidate whether CGRP or LTD₄ might affect airway and tissue responses differently, we measured resistance of airway and tissue using alveolar capsules (22).

Furthermore, we questioned whether and how CGRP or CGRP antagonist would modify hyperpnea-induced constriction, i.e., whether CGRP or CGRP antagonist would affect airway smooth muscle shortening or bronchial edema formation. To answer this question, we performed morphometric analysis.

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Am J Respir Crit Care Med Vol 154, pp 1551-1556, 1996

METHODS

Physiologic Preparation

Male Hartley guinea pigs ($n = 118$, 300 to 400 g) were studied. Animals were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and anesthesia was maintained with a dose of 10% of the initial dose every hour. Animals were tracheostomized and a metal cannula (internal diameter 2.1 mm, length 17 mm) was inserted into the trachea. Paralysis was induced with pancuronium bromide (0.8 mg/kg, intraperitoneally). Animals were mechanically ventilated (Model 683; Harvard Apparatus, South Natick, MA) at a frequency of 60 breaths/min, a tidal volume (V_T) of 9 ml/kg and positive end-expiratory pressure (PEEP) of 4 cm H_2O . Inspired gas was oxygenated, fully humidified and warmed to body temperature (1). The thorax was widely opened by means of a midline sternotomy. We used a heating pad to prevent cooling of the animals.

Two alveolar capsules were affixed to the pleural surface of the anterior portion of the lungs with cyanoacrylate. The pleura was punctured with an electrocautery needle through the central port of the capsule so as to bring the underlying alveoli into communication with the capsule chamber. A piezoresistive microtransducer (8507C-2; Endevco, San Juan Capistrano, CA) was placed in the capsule port to measure alveolar pressure (PA). Tracheal pressure (Ptr) was measured by a piezoresistive microtransducer (Endevco 8510B-2) placed in the lateral port of the tracheal cannula and tracheal flow was measured by means of a Fleisch pneumotachograph (no. 0000; Instrumentation Associates, New York, NY). Volume (V) was calculated by digital integration of the flow signal. All the transducers were calibrated simultaneously. All signals were amplified, filtered at a cutoff frequency of 100 Hz and converted by a 12-bit analog-digital converter (DT2801-A; Data Translation Inc., Marlborough, MA). The signals were sampled at a rate of 200 Hz and stored on an AT compatible computer.

Hyperpnea Challenge

At the start of the protocol, two deep inflations (3 times tidal volume, peak pressure of 30 cm H_2O) were performed to standardize volume history, and measurements of 10 s duration each were sampled three times as the baseline control. Then, the inspired gas was changed to a mixture of dry 95% O_2 /5% CO_2 at room temperature and the respiratory frequency was increased to 150 breaths/min (1). After hyperpnea challenge (HC) was performed for 7 min, the baseline ventilatory conditions were resumed, and measurements were recorded for up to 40 min.

Effects of CGRP(8-37) and CGRP on Hyperpnea-induced Constriction

30 minutes prior to HC, animals were intravenously pretreated with (1) saline as control (SAL, $n = 7$), (2) 0.1, 0.2, or 0.4 mg/kg CGRP(8-37) ($n = 6, 7, 6$, respectively), (3) 0.05, 0.1, or 0.2 mg/kg CGRP ($n = 6$, respectively). In the preliminary experiment, intravenous administration of greater than 0.3 mg/kg CGRP induced severe bradycardia or cardiac arrest, while 0.2 mg/kg or less dose of CGRP administration caused only transient bradycardia (1 to 2 min).

In another group ($n = 6$), we administered 0.2 mg/kg CGRP(8-37) and 0.2 mg/kg CGRP 2 min before HC in order to elucidate whether the receptor antagonist, CGRP(8-37), effectively blocks CGRP effects during hyperpnea-induced constriction. After HC, measurements were recorded for up to 40 min.

Effects of MK-571 and MK-886 on Hyperpnea-induced Constriction

30 minutes before HC, animals were intravenously pretreated with 1 mg/kg MK-571, or 2 mg/kg MK-886, ($n = 6$, respectively). In another group ($n = 6$), we administered 0.2 mg/kg CGRP(8-37) and 2 mg/kg MK-571 in combination 2 min before HC in order to elucidate whether CGRP would affect HC-induced constriction via LTD $_A$ -elicited responses. After HC, measurements were recorded for up to 40 min.

Effects of CGRP(8-37) and CGRP on LTD $_A$ -induced Constriction

To elucidate the roles of CGRP in LTD $_A$ -mediated responses, we studied whether CGRP and its antagonist modulate bronchoconstriction induced by exogenous LTD $_A$ administration. Animals were intravenously

pretreated with saline as control, 0.2 mg/kg CGRP(8-37), or 0.2 mg/kg CGRP ($n = 6$, respectively). Then, 3 μ g/kg LTD $_A$ was intravenously administered and measurements were made up to 15 min.

Effects of CGRP(8-37) and CGRP on Methacholine- and Endothelin-1-induced Constriction

We investigated the effects of CGRP(8-37) and CGRP on methacholine (MCh)-induced constriction to study the specificity of CGRP(8-37) and CGRP effects. Two minutes prior to the bolus of 10^{-7} mol/kg MCh, animals were pretreated with saline as controls ($n = 6$), 0.2 mg/kg CGRP(8-37) ($n = 5$), or 0.2 mg/kg CGRP ($n = 5$). Following the bolus of MCh, measurements were made up to 15 min.

In addition, the effects of CGRP(8-37) and CGRP on endothelin-1 (ET-1)-induced responses were studied. Two minutes prior to the bolus of 10^{-8} mol/kg ET-1, animals were pretreated with saline as controls ($n = 6$), 0.2 mg/kg CGRP(8-37) ($n = 5$), or 0.2 mg/kg CGRP ($n = 5$). Following the bolus of ET-1, measurements were made up to 15 min.

Calculation of Resistance and Elastance

The tracheal pressure was corrected for both the tube resistance and the Bernoulli effect. From flow, volume, and corrected Ptr, lung elastance (EL) and total lung resistance (RL) were calculated by fitting the equation of motion:

$$Ptr = RL \cdot (dV/dt) + EL \cdot V + K$$

where K was a constant term reflecting PEEP and the error linked to the residuals of least squares adjustment method. Tissue resistance (Rti) was calculated by fitting the equation of motion to PA:

$$PA = Rti \cdot (dV/dt) + EL \cdot V + K'$$

The values of RL and Rti were accepted only if differences in the values of EL obtained from Ptr and PA were less than 10% and both K and K' were less than 1 cm H_2O far from the real value of PEEP.

Airway resistance (Raw) was calculated by subtraction:

$$Raw = RL - Rti$$

where Rti was the average of the values obtained from two capsules.

Morphometric Study

In four animals from SAL, CGRP(8-37) (0.2 mg/kg), and CGRP (0.2 mg/kg) groups, morphometric examination of the lungs was performed. Based on the previous experiments (5, 20), it is anticipated that the peak response would occur approximately 10 min after cessation of the hyperpnea challenge. We therefore removed the lungs 10 min after challenge. Excised lungs were then frozen, submerged in liquid nitrogen. A constant transpulmonary pressure of 4 cm H_2O was maintained during freezing by delivering constant flow into the trachea. Frozen lungs were fixed in Carnoy's solution (60% ethyl alcohol, 30% chloroform, and 10% acetic acid) at $-70^\circ C$ for 18 h. Progressive concentrations of ethanol at $-20^\circ C$ were then substituted for the Carnoy's solution until 100% ethanol was reached. The tissue was maintained at $-20^\circ C$ for 4 h, warmed to $4^\circ C$ for 12 h and then allowed to reach and remain at room temperature for 2 h. After fixation, tissue blocks obtained from midsagittal slices of the lungs were embedded in paraffin. Blocks were cut 4 μ m thick using a microtome. Slides were stained with hematoxylin-eosin. We assessed tissue shrinkage and subsequent measurements were corrected for shrinkage.

Airway constriction was assessed by measuring the length of the epithelial basement membrane (Pbm) and the area (Abm) it circumscribed by projecting microscopic images onto a digitizer by means of a drawing attachment fixed to the microscope. The ideal area of the lumen of the relaxed airway (Abm*) was then calculated as:

$$Abm^* = Pbm^2/4\pi$$

and the degree of constriction (Abm/Abm^*) derived (23). The area circumscribed by the outer border of adventitia (Ao) was also measured and the area of the airway wall (WA) was calculated by the difference between Ao and Abm. We normalized WA to the relaxed area to adjust for differences in airway size. To assess whether airways were cut in cross section, the maximal diameter of the airway (D_1) and the diameter at the widest point perpendicular to this axis (D_2) were measured. We analyzed airways with a ratio of $D_2/D_1 > 0.33$.

Figure 1
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Raw =

% Change in R_L

Figure 2
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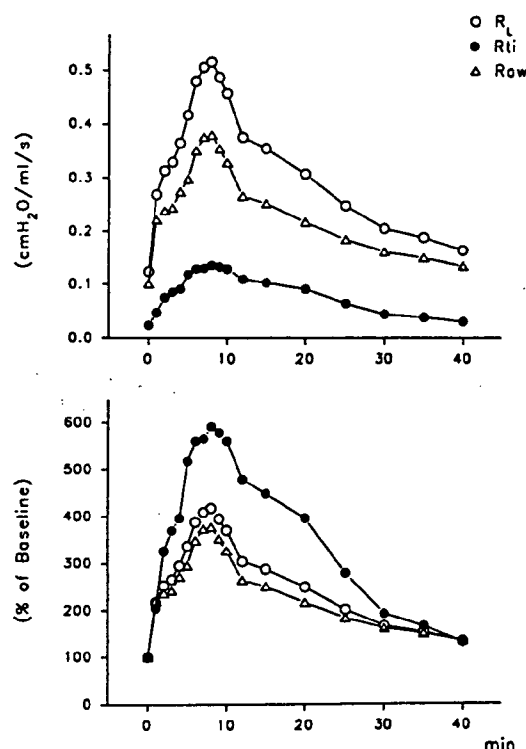


Figure 1. The time course of response in a hyperpnea-challenged animal. The peak response appears 8 min after the cessation of dry gas hyperpnea challenge. R_L = lung resistance; R_{ti} = tissue resistance; R_{aw} = airway resistance.

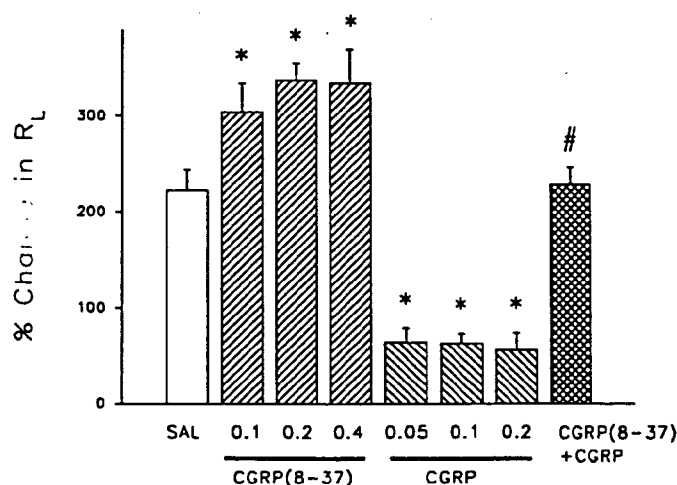


Figure 2. Percent changes in lung resistance (R_L) after dry gas hyperpnea challenge in the animals pretreated with saline (SAL), CGRP (8-37), CGRP, and 0.2 mg/kg CGRP(8-37) + 0.2 mg/kg CGRP. * p < 0.05 versus SAL group. # p < 0.05 versus CGRP groups. NS versus SAL group.

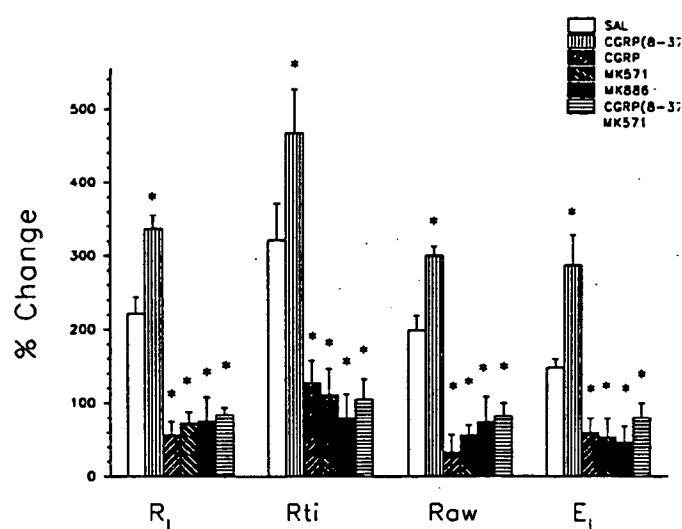


Figure 3. Percent changes in resistance of lung (R_L), tissue (R_{ti}), and airway (R_{aw}) and lung elastance (E_L) after dry gas hyperpnea challenge in the animals pretreated with saline (SAL), CGRP(8-37) (0.2 mg/kg), CGRP (0.2 mg/kg), MK-571 (2 mg/kg), MK-886 (2 mg/kg), and CGRP(8-37) (0.2 mg/kg) + MK-571 (2 mg/kg). * p < 0.05 versus SAL group.

Lung tissue distortion was assessed by measuring the mean linear intercept between alveolar walls (L_m) (24) and the standard deviation of L_m (SD L_m) in 20 randomly selected nonoverlapping fields. SD L_m was calculated using the 20 values of interalveolar wall distance obtained from the 20 fields. Measurements were made using a microscope with a 10 \times objective lens and a 10 \times eyepiece.

Data Analysis

Comparisons of physiologic and morphometric data among each group were performed using analysis of variance test. P values less than 0.05 were taken as significant and data are expressed as mean \pm SE.

RESULTS

Physiologic Study

Figure 1 demonstrates the typical time course of response in a single animal. The peak response was observed in this animal at 8 min after the cessation of hyperpnea challenge.

The effects of CGRP(8-37) and CGRP on HC-induced responses are summarized in Figure 2. Pretreatment with CGRP reduced HC-induced responses, while antagonism of CGRP receptor using CGRP(8-37) enhanced HC-induced changes. As shown in Figure 2, 0.2 mg/kg CGRP(8-37) effectively blocks the effects of CGRP on HC-induced constriction.

Table 1 shows baseline values of R_L , R_{ti} , R_{aw} , and E_L in the SAL, CGRP(8-37) (0.2 mg/kg), CGRP (0.2 mg/kg), MK-571, MK-886, and CGRP(8-37) + MK-571 groups. There were no significant differences in these variables among the groups. The results of the physiologic study are summarized in Figure 3.

TABLE 1
BASELINE MEASUREMENTS

	SAL	CGRP (8-37) (0.2 mg/kg)	CGRP (0.2 mg/kg)	MK-571	MK-886	CGRP (8-37) + MK-571
R_L , cm H_2O /ml/s	0.115 \pm 0.007	0.116 \pm 0.010	0.113 \pm 0.007	0.122 \pm 0.009	0.121 \pm 0.008	0.115 \pm 0.008
R_{ti} , cm H_2O /ml/s	0.027 \pm 0.003	0.027 \pm 0.003	0.032 \pm 0.004	0.032 \pm 0.003	0.030 \pm 0.001	0.031 \pm 0.003
R_{aw} , cm H_2O /ml/s	0.088 \pm 0.009	0.089 \pm 0.008	0.082 \pm 0.009	0.091 \pm 0.009	0.092 \pm 0.007	0.085 \pm 0.008
E_L , cm H_2O /ml	1.89 \pm 0.31	1.71 \pm 0.37	1.90 \pm 0.13	2.02 \pm 0.19	1.92 \pm 0.29	1.85 \pm 0.22

Definition of abbreviations: R_L = lung resistance; R_{ti} = tissue resistance; R_{aw} = airway resistance; E_L = lung elastance; SAL = saline.

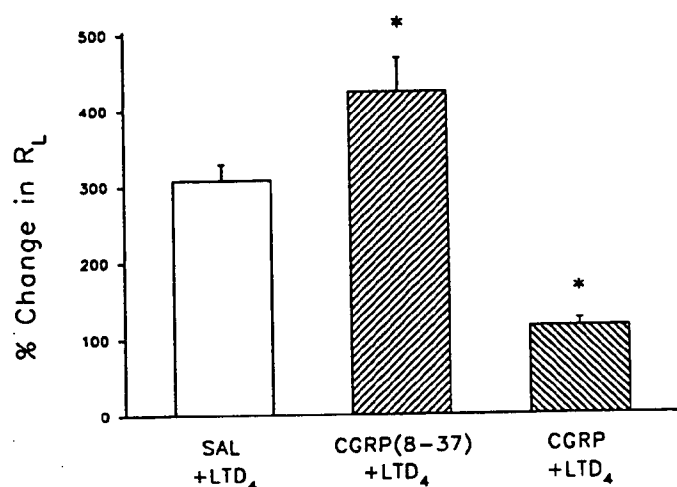


Figure 4. Percent changes in lung resistance (R_L) after 3 μ g/kg leukotriene D₄ intravenous administration in the animals pretreated with saline (SAL), 0.2 mg/kg CGRP(8-37) and 0.2 mg/kg CGRP. * $p < 0.05$ versus SAL control group.

Pretreatment with either MK-571 or MK-886 significantly reduced changes in R_L , R_{ti} , R_{aw} , and E_L compared with saline control. Pretreatment with CGRP(8-37) and MK-571 in combination also significantly attenuated HC-induced responses.

The effects of CGRP(8-37) and CGRP on LTD₄-induced constriction are shown in Figure 4. Pretreatment with CGRP reduced LTD₄-induced constriction, whereas antagonism of CGRP increases the response elicited by LTD₄ administration. As shown in Figure 5, neither CGRP(8-37) nor CGRP had significant effects on MCh- and ET-1-induced constriction, suggesting that the effects of CGRP might be specific for LTD₄ modulation.

Morphometric Study

Table 2 summarized the morphometric data. There were no significant differences in Pbm and D₂/D₁ among the SAL, CGRP(8-37), and CGRP groups. These results indicate that there were no significant biases among the three groups in terms of airway selection. There were significant differences in Abm/Abm* and SDLm between the SAL and CGRP(8-37) groups and between the SAL and CGRP groups. These findings suggest that CGRP(8-37) increases the degree of airway narrowing and tissue distortion and that CGRP attenuates hyperpnea-induced responses. WA/Abm* in the CGRP group was significantly greater than that in the SAL group, suggesting that CGRP may increase airway wall thickness compared with the control.

Figure 6 demonstrates photomicrographs of representative airways and tissues from each group. In a SAL and a CGRP(8-37) group animal, substantial degrees of airway narrowing and lung tissue distortion were observed. Airway constriction and parenchymal heterogeneity were moderate in samples from a CGRP group animal.

DISCUSSION

The results of the current study show that CGRP and LTD₄ are involved in the pathophysiology of hyperpnea-induced constriction in guinea pigs. The administration of CGRP or antagonism of LTD₄ reduced HC-induced changes in both airway and tissue resistance, whereas CGRP antagonist, i.e., CGRP(8-37), enhanced HC-induced constriction compared with control. Pretreatment with CGRP(8-37) and MK-571 in combination significantly at-

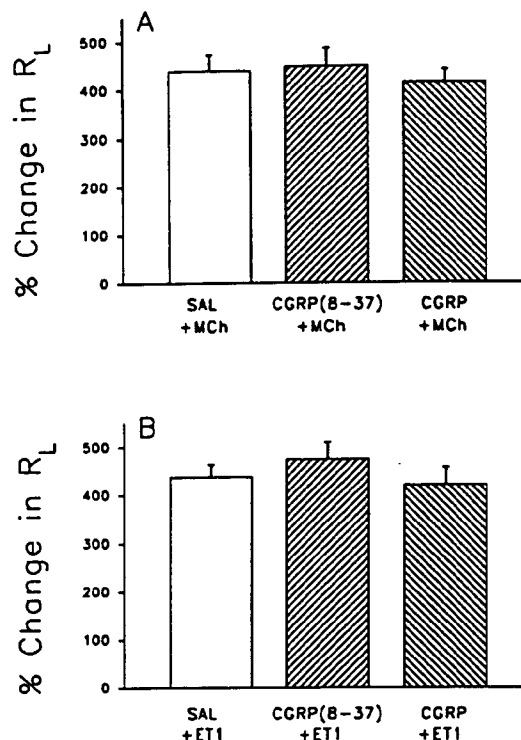


Figure 5. (A) Effects of CGRP(8-37) (0.2 mg/kg) and CGRP (0.2 mg/kg) on methacholine (MCh, 10^{-7} mol/kg) induced constriction. (B) Effects of CGRP(8-37) (0.2 mg/kg) and CGRP (0.2 mg/kg) on endothelin-1 (ET-1, 10^{-8} mol/kg) induced constriction. R_L = lung resistance; SAL = saline.

tenuated HC-induced responses, suggesting that CGRP might affect HC-induced constriction via LTD₄ responses. In addition, pretreatment with CGRP reduced LTD₄-mediated responses. These observations suggest that CGRP may have an inhibitory effect on dry gas hyperpnea-induced constriction in guinea pigs via modulation of LTD₄-elicited responses.

Hyperpnea-induced bronchoconstriction in tracheostomized, mechanically ventilated guinea pigs resembles characteristics of human exercise-induced asthma in several ways (1-5, 25). (1) The onset of constriction occurs a few minutes after, not during, dry gas hyperpnea challenge, and resolves spontaneously (Figure 1). (2) The response to hyperpnea challenge is reproducible follow-

TABLE 2
MORPHOMETRIC RESULTS

	SAL	CGRP (8-37) (0.2 mg/kg)	CGRP (0.2 mg/kg)
Number of airways	73	74	73
Per animals	18.3 ± 0.9	18.5 ± 0.3	18.3 ± 0.5
Pbm, mm	1.045 ± 0.063	1.042 ± 0.060	1.036 ± 0.044
D ₂ /D ₁	0.750 ± 0.016	0.740 ± 0.013	0.765 ± 0.018
Abm/Abm*	0.392 ± 0.025	0.178 ± 0.013†	0.693 ± 0.020†
WA/Abm*	0.327 ± 0.018	0.302 ± 0.019	0.406 ± 0.015†
Lm, μ m	65.8 ± 1.1	64.4 ± 1.1	66.9 ± 1.2
SDLm, μ m	23.8 ± 2.6	32.8 ± 2.1†	11.1 ± 1.0†

Definition of abbreviations: Pbm = length of basement membrane; D₂/D₁ = index of airway roundness; Abm* = ideally relaxed area; Abm/Abm* = degree of airway constriction; WA = airway wall area; Lm = mean linear intercept; SDLm = standard deviation of Lm; SAL = saline.

† $p < 0.01$ versus SAL group.

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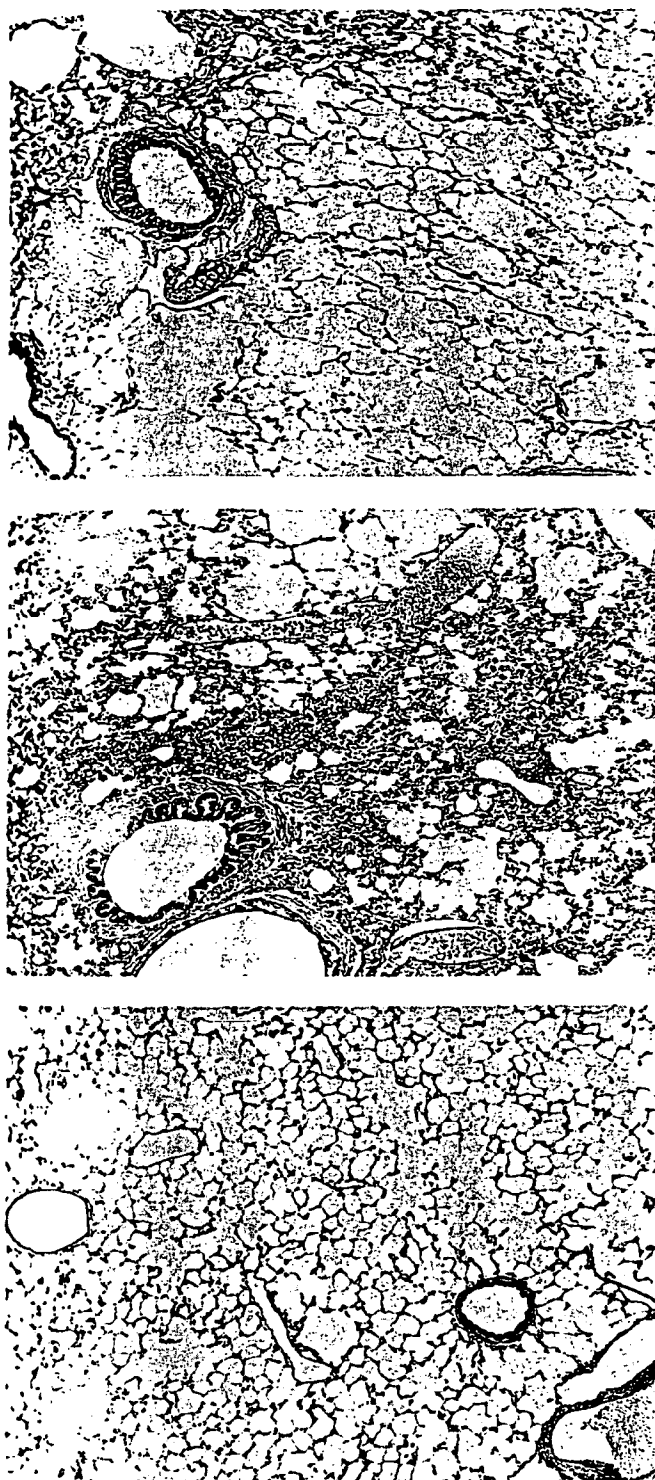


Figure 6. Photomicrograph of airways and tissues from the animals pretreated with saline (top), 0.2 mg/kg CGRP(8-37) (middle), and 0.2 mg/kg CGRP (bottom). Hematoxylin-eosin stain; magnification: $\times 40$.

ing consecutive identical challenge. (3) Warming and humidifying inspired gas diminishes the response. (4) There are relationships between the amount of hyperpnea and the degree of response elicited. It has been reported that hyperpnea-induced bronchoconstriction in guinea pigs is mediated through tachykinin release from airway sensory nerves, because tachykinin depletion through capsaicin pretreatment attenuates the response (6). However, it

remains to be clarified whether another sensory neuropeptide, CGRP, may have a potential role in hyperpnea-induced bronchoconstriction.

CGRP, a 37 amino acid peptide, is made by sensory C fibers throughout the respiratory tree. It has been shown that CGRP, a potent vasodilator (26), modulates hypoxic pulmonary vasoconstriction (27), while CGRP receptors have been found to densely populate lung vessels (28). In the present study, the administration of CGRP attenuated HC-induced constriction, whereas antagonism of CGRP with CGRP(8-37) enhanced the response to dry gas hyperpnea. These observations suggest that CGRP might have an inhibitory role in the pathophysiology of dry gas hyperpnea-induced constriction in guinea pigs. In addition, we observed that pretreatment with CGRP(8-37) and MK-571 in combination attenuated HC-induced responses and that pretreatment with CGRP reduced LTD₄-induced constriction, suggesting that CGRP may modulate LTD₄-mediated responses and attenuate HC-induced constriction in guinea pigs. Based on the observations that neither CGRP(8-37) nor CGRP had significant effects on the constriction induced by MCh or ET-1, the effects of CGRP might be specific for LTD₄ modulation.

Potent anti-inflammatory action of CGRP has recently been reported. Raud and colleagues (10) have demonstrated that CGRP, but not substance P, inhibits edema-promoting actions of inflammatory mediators *in vivo* in rodents and humans. It has been shown by Di Marzo and coworkers (9) that CGRP inhibits the release of leukotrienes including LTC₄ and LTD₄ from platelet-activating factor-stimulated rat lungs and ionophore-stimulated guinea pig lungs. The mechanism of the inhibitory effects of CGRP on HC-induced constriction deserves consideration. The exogenous administration or the endogenous release of CGRP might inhibit the effects of leukotrienes, resulting in the attenuation of HC-induced constriction. Another potential mechanism is that CGRP might affect HC-induced constriction via vasodilating effects (26). Recently, it has been postulated by Ray and coworkers (29) that local heat/water loss relates to HC-induced constriction in guinea pigs. By causing vasodilation, CGRP could affect heat and water transfer through the bronchial mucosa during HC. Of note, in the current study, airway wall area in the CGRP-treated group was greater than that in the control group, suggesting that CGRP might cause changes in water balance in airway wall.

Both MK-571 (a LTD₄ receptor antagonist) and MK-886 (a 5-lipoxygenase inhibitor) inhibited hyperpnea-induced bronchoconstriction, suggesting that leukotrienes have an important role in the pathophysiology of dry gas hyperpnea-induced constriction in guinea pigs. In humans, it has been demonstrated that LTD₄ receptor antagonists inhibit exercise-induced bronchoconstriction (30) and that inhibition of 5-lipoxygenase by leukotriene synthesis inhibitors is associated with a significant amelioration of the asthmatic response to cold, dry air (31). In guinea pigs, Garland and coworkers (7) have shown that hyperpnea-induced bronchoconstriction was 50 to 80% reduced by either LTD₄ receptor antagonist or 5-lipoxygenase inhibitor, which was confirmed by the present study. Recently, it has been suggested that LTD₄ might relate to the release of tachykinin-like substances from airway sensory nerves (32). Potentially, LTD₄ could be involved in the pathogenesis of HC-induced constriction as a key mediator.

In the control hyperpnea-challenged group, both Raw and Rti markedly increased after dry gas hyperpnea challenge as previously reported (20). CGRP reduced HC-induced responses in airway and lung tissue similarly, whereas CGRP antagonist increased both Raw and Rti. LT antagonists also decreased both airway and lung tissue responses to HC. It seems that there is no difference in the susceptibility of Raw and Rti to the effects of CGRP or LTD₄.

Morphometric results including the degree of bronchoconstriction and parenchymal distortion were compatible with physiologic observations. The remarkable finding is that the airway wall area in the CGRP group significantly increased compared with the controls, while the degree of airway constriction in the CGRP group was markedly smaller than that in the controls. These observations may suggest that CGRP attenuates HC-induced constriction by reducing airway smooth muscle shortening. In contrast, CGRP may increase bronchial edema or hyperpnea-induced bronchovascular hyperpermeability.

Finally, in the current study, the stability of CGRP and CGRP(8-37) in plasma remains to be clarified. In humans, Zaidi and colleagues (33) have reported that the half-life of CGRP for biologic activity is estimated at 19 min. If the biologic activity for CGRP and CGRP(8-37) would be insufficient due to the lack of stability in the present protocol, one might assume that the metabolites of CGRP or CGRP(8-37) would also affect HC-induced responses, based on the results of CGRP and CGRP(8-37) study. It seems unlikely, however, that loss of the stability of CGRP and CGRP(8-37) would be of sufficient magnitude to invalidate the interpretation of the current results.

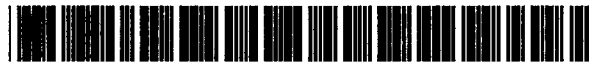
In conclusion, we demonstrated that administration of CGRP, MK-571, and MK-886 reduced hyperpnea-induced responses and that CGRP(8-37), CGRP antagonist, enhanced hyperpnea-induced constriction, suggesting that CGRP and LTD₄ could be involved in the pathophysiology of hyperpnea-induced constriction in guinea pigs. In addition, pretreatment with CGRP(8-37) and MK-571 in combination attenuated HC-induced responses and CGRP attenuated LTD₄-induced constriction in guinea pigs. These observations suggest that CGRP might modulate LTD₄-elicited responses and could have inhibitory effects on dry gas hyperpnea-induced constriction in guinea pigs, an animal model of exercise-induced asthma in humans.

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Appendix E
U.S. Patent No. 5,635,478



US005635478A

United States Patent [19]
Vignery[11] **Patent Number:** **5,635,478**
[45] **Date of Patent:** **Jun. 3, 1997**[54] **USE OF CALCITONIN GENE-RELATED
PEPTIDE TO REGULATE IMMUNE
RESPONSE**[75] **Inventor:** **Agnès M.-C. Vignery**, New Haven,
Conn.[73] **Assignee:** **Yale University**, New Haven, Conn.[21] **Appl. No.:** **125,275**[22] **Filed:** **Sep. 23, 1993****Related U.S. Application Data**

[63] Continuation of Ser. No. 408,573, Sep. 18, 1989, abandoned.

[51] **Int. Cl.⁶** **A61K 38/00**; C07K 5/00;
C07K 7/00[52] **U.S. Cl.** **514/12**; 530/324[58] **Field of Search** 514/12; 530/324[56] **References Cited**
PUBLICATIONSNong et al. "Peptides Encoded by the Calcitonin Gene
Inhibit Macrophase Function", J. Immun., 143, 45-49 (Saly
1989).*Primary Examiner*—Avis M. Davenport*Attorney, Agent, or Firm*—Millen, White, Zelano, & Brani-
gan, P.C.[57] **ABSTRACT**Calcitonin gene-related peptide regulate immune cell func-
tion and cytokine release and are useful in the treatment of
immune cell and cytokine mediated, immune mediated
diseases, such as rheumatoid arthritis, treating viral
infections, tumors and organ transplants.**9 Claims, No Drawings**

USE OF CALCITONIN GENE-RELATED PEPTIDE TO REGULATE IMMUNE RESPONSE

This is a continuation, of application Ser. No. 07/408,573 filed Sept. 18, 1989 and now abandoned.

This invention relates to a method of regulating the immune response with calcitonin gene-related peptide (CGRP) and to the treatment of various diseases and abnormal conditions associated with the immune response.

BACKGROUND OF THE INVENTION

Macrophages and lymphocytes play a central role in inflammatory reactions in response to foreign or infectious agents. Monocytes are first chemotactically attracted to the injured tissue in which they differentiate structurally and functionally into tissue specific macrophages. Via the expression of specific receptors and the release of appropriate cytokines, macrophages govern and coordinate in a well ordered series of cellular mediated events the inflammatory reaction which eventually leads to the eradication of the intruder and the repair of the damaged tissue. When inflammation persists and becomes chronic, macrophages that have been accumulating fuse to form multinucleated giant cells onto the foreign substrate. Although these giant macrophages appear to be actively involved in tumor defense mechanisms and bone resorption (where they are called osteoclasts), the functional relevance of their multinucleation remains highly speculative. Earlier reports indicate that giant cells can phagocytose as effectively as macrophages through a variety of receptors. This suggests that macrophages and giant cells share functional components and are equally capable of participating in host defense mechanisms. It has previously been shown that giant cells, whether elicited *in vivo* or *in vitro* are, like osteoclasts, polarized and express antigens that are not detected in mononucleated macrophages. To further investigate the role played by giant cells in chronic inflammatory reactions and to elucidate the putative interactions between giant cells and/or osteoclasts and the surrounding immune and nonimmune cells, the level of release of two cytokines, IL-1 and IL-6, by macrophages was compared to that of multinucleated giant cells. Because osteoclast activity is strongly inhibited by CT, the effect of this hormone was investigated to determine whether it could alter the level of release of these cytokines by multinucleated macrophages. This investigation demonstrated that giant cells, like peritoneal macrophages, do release spontaneously detectable levels of both IL-1 and IL-6; that CT is a weak inhibitor of IL-1 release but is specific for giant cells; that CGRP, a neuropeptide encoded by the same gene as CT, specifically blocks the release of IL-1 in both peritoneal macrophages and multinucleated giant cells; and that this inhibitory action of CGRP is mediated by intracellular cyclic adenosine monophosphate (cAMP) dependent mechanism. This work also demonstrates that CGRP directly alters the pattern of cytokine release by lymphocytes and has a profound effect on cytokine releasing cells (keratinocytes, mesangial cells, glial cells, etc.).

In a recently published paper, Nong, Yu-Hua et al., *J. Immun.*, 143, 45-49, No. 1, pp. 45-49 (Jul. 1, 1989), report that CGRP profoundly inhibit the ability of macrophages to produce H_2O_2 in response to IFN or to act as APC and that CT also prevented macrophage activation, suggesting to the authors that CGRP and CT play an important role in modulating the ability of macrophages to present Ag and to respond to activating factors.

SUMMARY OF THE INVENTION

In a method aspect, this invention relates to a method of regulating the function of macrophages and lymphocytes releasing cells, respectively, in a living mammal which comprises administering thereto an immune cell function-inhibiting amount of CGRP.

In another method aspect, this invention relates to a method of regulating the rate of cytokine release from giant cells in a living mammal which comprises administering thereto an IL-1 or IL-1 and IL-2, respectively release-inhibiting amount of CT.

In a preferred aspect, this invention relates to a method of treating an immune response-associated disease or immune response-mediated abnormal condition in an animal by the administration thereto of an amount of CGRP effective to ameliorate the disease or abnormal condition.

In another preferred aspect, this invention relates to a method of treating an immune response-associated disease or immune response-mediated abnormal condition in an animal by the administration thereto of an amount of CT effective to ameliorate the disease or abnormal condition.

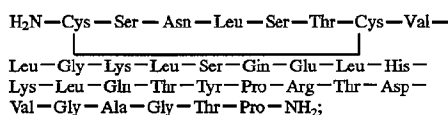
In another method aspect, this invention relates to a method for the symptomatic treatment of a disease resulting from a viral infection in an animal which comprises administering systemically to the infected animal successive therapeutically effective doses of CGRP or CT.

In a composition aspect, this invention relates to a pharmaceutical composition adapted for noningestion systemic administration thereof comprising an immune cell function-inhibiting amount per unit dosage of CGRP in admixture with a pharmaceutical carrier adapted for noningestion systemic administration.

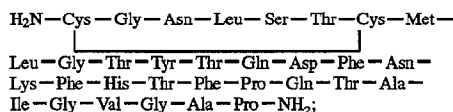
DETAILED DISCUSSION

CGRP and CT are known commercially available peptides.

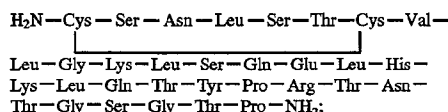
Eel calcitonin has the formula



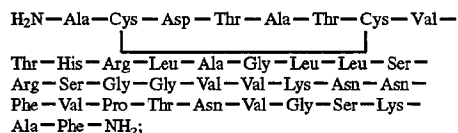
Human CGRP has the formula



Salmon 1 calcitonin has the formula



Human CGRP has the formula



CGRP and CT are useful in the treatment of a variety of ailments and diseases in animals, particularly those which result in inflammatory and related stress conditions manifesting themselves in the afflicted animal. For example, CGRP and CT are useful in relieving the pain, tenderness, fever and dysfunction following acute traumatic injuries, surgery and in the treatment of orthopedic dysfunction, e.g., bony exostosis. CGRP also is effective in treating viral diseases, e.g., human influenza A and B, viral horse pneumorhinitis, canine distemper, picorna virus induced feline pneumotracheitis, dysfunctions based on the family of herpes virus and diseases associated with H.I.S. CGRP and CT are useful in the treatment of a variety of acute and chronic inflammatory conditions. Its anti-inflammatory activity is manifested in various animal models of induced

inflammation, viz., foot paw edema in the rat produced by carrageenin, yeast or silver nitrate; adjuvant-induced polyarthritis in the rat; passive cutaneous arthritis reaction; cotton pellet granuloma in the nonadrenalectomized and bilaterally adrenalectomized rat; pox virus-induced skin edema in the rabbit; PVA sponge implant-induced inflammation and wound healing and antiserum-induced skin edema and active anaphylaxis in the guinea pig and the mouse.

Among the inflammatory conditions for which CT and CGRP are useful in the treatment thereof, are those in which synthetic anti-inflammatory agents have limited utility, e.g., because of toxic side effects upon prolonged use.

More specifically, CT and CGRP are useful in ameliorating inflammatory conditions and mitigating the effects thereof, for instance those involving the urinary tract and the joints, in various mammals. It is useful in alleviating the symptoms of and the structural deformities associated with post-traumatic arthritis and rheumatoid diseases, such as bursitis, tendonitis, osteoarthritis, nonsurgical disc syndrome and myositis.

CGRP and CT are also useful in the treatment of diseases involving an imbalance of the auto-immune system, alone and in combination with drugs conventionally used to treat such diseases. Typical are the "collagen" type diseases, e.g., rheumatoid arthritis, lupus erythematosus and scleroderma, allergic states, e.g., penicillin reaction, which are characterized by multiple wheals, indurations, erythemas, edema or itching, and drug-induced photosensitization.

In addition to its anti-inflammatory effects, CGRP and CT protect from shock reactions produced upon antigenic challenge after prior sensitization.

CGRP and CT can be used in conjunction with accepted forms of therapy and medication, e.g., hormonal, including androgen and estrogen, therapy.

CGRP and CT also can be used concurrently or alternatively with steroids in anti-inflammatory therapy, e.g., with cortisone, hydrocortisone, prednisone, prednisolone, and the corresponding $\Delta^{1,4}$ -9 α -fluoro-16-hydroxy, 16 α -methyl and 16 β -methyl substituted steroids, e.g., dexamethasone, fluorocortisone, fluoromethalone, methylprednisolone, triamcinolone and its acetonide, betamethasone, and their known esters and derivatives, and nonsteroid anti-inflammatory agents, e.g., acetylsalicylic acid, salicylamide, aminopyrine, chloroquine, hydroxychloroquine, phenylbutazone and indomethacin. CGRP and CT can also be used concurrently or alternately with known agents used in antibacterial and in anti-viral therapy to increase the effectiveness of conventional dosages of the known agents or, by reducing such dosages of such agents, the toxic and side effects ordinarily associated with such therapy.

The pharmaceutical compositions of this invention comprise CGRP and/or CT and a pharmaceutically acceptable carrier. The form and character which this carrier takes is, of course, dictated by the mode of administration.

Oral administration, e.g., sublingual, is possible, particularly if the peptide is protected from the destructive action of the acid pH and enzymes of the stomach, e.g., in the form of an enteric coated tablet, although much larger doses are generally required by this route. CGRP has topical activity, e.g., when applied as a solution, aerosol, cream, ointment, salve, etc., which renders it useful for treating corneal and conjunctival, respiratory, genito-urinary and dermatological disorders. Desirably, it is administered with a surfactant and/or penetrant to ensure better contact and penetration.

The pharmaceutical compositions can, e.g., be in a form of pills, dragees and tablets, provided they are coated by

known techniques to delay disintegration and absorption in the gastro-intestinal tract and to protect the CGRP or CT from stomach acid and enzymes.

Aqueous solutions contain CGRP or CT in admixture with excipients suitable for the manufacture of stable aqueous solutions, e.g., NaCl to provide a saline or isotonic solution, buffer agents, acids or bases, etc. The aqueous solution can also contain one or more preservatives, for example, ethyl or n-propyl p-hydroxybenzoate.

Storage-stable compositions can be produced in accordance with the methods of U.S. Pat. No. 3,637,640 and the prior art cited therein, whose disclosures are incorporated herein by reference.

Oily suspensions may be formulated by suspending CGRP or CT in an oil suitable for injection or topical administration, in a vegetable oil, e.g., arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil, e.g., a liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. These compositions may be preserved by the addition of an antioxidant, e.g., ascorbic acid.

The pharmaceutical compositions of the invention can be in the form of oil-in-water emulsions suitable for parenteral administration. The oily phase may be a vegetable oil, e.g., olive oil or arachis oil, or a mineral oil, e.g., liquid paraffin or mixtures of these. Suitable emulsifying agents are naturally occurring gums, e.g., gum acacia or gum tragacanth, naturally occurring phosphatides, e.g., soya bean lecithin and esters of partial esters derived from fatty acids and hexitol anhydrides, for example, sorbitan monooleate, and condensation products of the said partial esters which ethylene oxide, for example, polyoxy-ethylene sorbitan monooleate.

The compositions of this invention can also be in the form of an aerosol for inhalation or topical administration or slow-dissolving pellets for implantation.

The compositions of this invention can be administered parenterally or topically. The term parenteral as used herein includes subcutaneous, intradermal, intravenous, intramuscular, intraocular, intrastromal, intrasynovial, intrathecal, intramural, intraarticular, intraperitoneal, intrascrotal, intraosseous, intraspinal, intraligamentous and intrasternal. Intramuscular, and subcutaneous administration is usually preferred except when the CGRP or CT is administered proximate a localized area of inflammation.

The pharmaceutical compositions can be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous solution. The solution can be formulated according to the known art using those carriers mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, e.g., 1,3-butanediol.

The compositions of this invention can be in the form of suppositories for vaginal and rectal administration. These compositions can be prepared by mixing CGRP or CT with a suitable nonirritating excipient which is solid at ordinary temperatures but liquid at body temperature and will therefore melt in the rectum or vagina to release the drug, e.g., cocoa butter and polyethylene glycols.

The compositions of this invention combine an effective unit dosage amount of CGRP and/or CT, i.e., either is present at a concentration effective to evoke the desired response when a unit dose of the composition is administered by the route appropriate for the particular pharmaceutical carrier. For example, liquid compositions, both topical and injectable, usually contain about 0.5 to 100 mg of the peptide per 0.25 to 10 cc, preferably about 0.5 to 5 cc, except

I.V. infusion solutions, which can also be more dilute, e.g., 0.5 to 20 mg of the peptide per 50–1,000 ml, preferably 100–500 ml of infusion solution. Tablets, capsules and suppositories usually contain 1.0 to 500 mg, preferably 10 to 100 mg, per unit dosage.

The weight ratio of CGRP or CT to liquified propellant in an aerosol for topical or inhalation administration can be quite high, e.g., 0.5–5%. Topical compositions usually contain one of the peptides in a concentration of 0.1–1% in aqueous solution or nonaqueous suspension.

The amount of either peptide administered is dependent on several factors, including the species of patient, the condition of the patient prior to the peptide therapy, the particular disease and its progression and the route of administration. The usual individual parenteral dose range of the peptide is about 10 mg to 100 mg, usually 25 mg to 75 mg. The size of an individual dose is primarily dependent upon the dynamics of the disease pattern. For instance, in an acute stage of a disease, e.g., with associated toxemia or uremia, injections spaced about every 6 hours may be required, with the frequency subsequently reduced to 8–12 hours and then every 24 hours or longer, depending on the clinical picture. Thus, during the acute state of a disease, the frequency of the injections is often more critical than the amount of each individual dose.

Larger individual doses are usually administered when the peptide is administered orally, e.g., 5 mg, 25, 50 or 100 mg, or even more. Similarly, when a solution or suspension of the peptide is applied topically to the skin or infused into the bladder, vagina, large intestine, etc., the total amount of peptide administered in single uninterrupted dose can vary from 5 to 100 mg or more. Conversely, when the peptide is administered into the respiratory tract, e.g., in the treatment of asthma, anaphylactic or other acute shock conditions, e.g., as a spray, mist, aerosol, etc., lesser amounts, e.g., 0.5 to 25 mg or less may be indicated.

The spacing of the individual doses is also partially determined by the nature of the ailment. In treatment of inflammatory syndromes, the selected peptide is usually administered in multiple successive dosages, spaced as frequently as 6–12 hours apart and as long as six weeks apart. Usually, daily doses are administered until symptomatic relief, e.g., from pain and stiffness, is obtained. Thereafter, doses are spaced further apart, the frequency being adjusted so that recurrence of symptoms is avoided and relief maintained. Treatment can be continued over a period of several weeks or months, and indefinitely for advanced chronic cases.

In treating viral infections, the selected peptide is initially administered in multiple successive dosages usually spaced every 12 to as frequently as every 4 hours.

CGRP and CT are usually administered by instillation or by injection, e.g., intramuscularly, subcutaneously, intravenously or intradermally. I.M. is preferred, except in acute situations, where I.V. is sometimes preferred for more rapid onset of effect, and in certain localized disorders where local injection may be more effective. Individual doses usually fall within the range of 10–100 mg. The preferred dosage for humans is about 50 mg. The exact dosage is not critical and depends on the type and the severity of the disease.

Contemplated equivalents of this invention are methods and compositions wherein the CGRP is replaced by a structurally related peptide which possesses the amino acid spatial configuration of CGRP which is responsible for its IL-1 release inhibiting activity, which configuration can be determined by conventional structure-activity peptide analysis.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight.

The entire texts of all applications, patents and publications cited herein are hereby incorporated by reference.

Material and Methods

Cells.

Rat alveolar and peritoneal macrophages were obtained from 12 week old Fisher 344 rats (Charles River, Kingston, N.Y.). Alveolar macrophages were collected by tracheobronchial lavage as previously described (Vignery et al., J. Histochem Cytochem., 1989). Peritoneal macrophages were collected from the same rats prior to the lung lavage. The collected cells were: (i) washed twice in Minimum Essential Medium with Earle's salts (MEME) supplemented with 2 mM L-glutamine and vitamins; (ii) plated in 6 well dishes at 10^7 cells/ml, 10^6 cells per well, in MEME containing 10% heat inactivated human serum (HS); (iii) incubated for 20 minutes at 37° C. in 5% CO₂, 95% air to let the macrophages adhere; (iv) subsequently grown at 10^6 cells/ml of MEME containing 5% heat inactivated HS. Cells were cultured in FCS for the IL-6 bioassay. All tissue culture reagents were purchased from Gibco Laboratories, Grand Island, N.Y. The adherent cell populations were composed of 99% macrophages and a few occasional polymorphonuclear cells as determined by Wright stain.

Interleukin-1 and Interleukin-6 Release Macrophage supernatants.

To estimate the release of IL-1 and IL-6 by peritoneal macrophages and fused alveolar macrophages, the cells were collected and cultured as described above. Prior to perform the release, the cells were washed twice with warm MEME and subsequently incubated for 24 hours in 1 ml of MEME supplemented with 5% of HS or FCS, with or without agonist. At the termination of the release, the supernatant were collected, spun at 400 g for 10 minutes, transferred to microfuge tubes and stored at –20° C. until assayed. Macrophages remained adherent and viable in all experiments as determined by DNA measurements and trypan blue exclusion.

IL-1 Bioassay.

IL-1 activity in the macrophage supernatants was measured according to Gillis and Mizel (Proc. Natl. Acad. Sci., USA, 1981, 78:1133–1137). LBRM-33 cells, clones 1A5 and 5A4 (ATCC, Rockville, Md.) were stimulated with either PHA (Gibco), rm IL-1 β or log₂ dilutions of the macrophage supernatants. LBRM-33 lymphocyte supernatants were harvested from 24 hour cultures and subsequently tested for IL-2 activity using a standard microassay, Gillis et al. (J. Immunol., 1978, 120: 2027–2032), based on the IL-2 dependent exponential proliferation of a murine cytotoxic T-cell line CTLL (ATCC, Rockville, Md.), Gillis and Smith (Nature (London), 1977, 268: 154–156). CTLL cells were cultured in the presence of a log₂ dilution series of putative IL-2 containing samples. After 24 hours, the cells were exposed to 0.5 μ Ci [³H]dThd (20 mCi/mmol, New England Nuclear) for an additional 4 hours after which the cultures were harvested onto glass fiber filter strips with the aid of a multiple automated sample harvester (MASH 11, Microbio-

logical Associates, Bethesda, Md.). [^3H]dThd incorporation was determined by direct counting.

IL-6 Bioassay.

Five thousand B13.29 cells subclone B9 (donated by Dr. Thomas Kupper, Washington University of School of Medicine, Washington, Mo.) were cultured in the presence of a log₂ dilution series of macrophage supernatants or standard dilutions of rmIL-6 according to Aarden, L. A. (Eur. J. Immunol., 1987, 17: 1411-1416). Cells were labeled after 24 hours for 1 hour with 0.2 μCi [^3H]dThd (20 mCi/mmol, New England Nuclear).

Generation of Intracellular cAMP.

For cAMP studies, cells were grown under the conditions described above but using either 24 or 96 well dishes. Each well contained either 2×10^5 or 5×10^4 cells grown in 800 or 200 μl , respectively. The cells were first washed with warm MEME and subsequently incubated at 37° C. in MEME supplemented with BSA (1 mg/ml)(Sigma Chemical, St. Louis, Mo.) either in the absence or presence of Salmon Calcitonin (sCT)(kindly provided by Dr. Orlowski, Rorer Central Research, Horsham, Penn.), rat calcitonin gene-related peptide (CGRP)(Bachem, Inc., Torrance, Calif.) and 3-isobutyl-1-methylxanthine (IBMX)(Sigma) for the indicated times. Incubations were terminated by aspirating the medium and adding 100 μl of 0.4% perchloric acid to each well. Perchloric acid extracts were neutralized with 1.5M KOH and stored at -20° C. These samples were acetylated and cAMP measured by radioimmunoassay using succinylated ^{125}I -labeled cAMP (Biomedical Technologies, Inc., Stoughton, Mass.). Results were expressed as femtomoles of cAMP per well. Experiments were performed in duplicates or triplicates and repeated at least 3 times.

DNA Measurements.

DNA was measured using a modification of the technique described by Labarca and Paigen (Anal. Biochem., 1980, 102: 344). In brief, plated cells were quickly rinsed with PBS prior to a 20-minute incubation at 37° C. in 200 μl of 10 mM EDTA pH 12.3. The cells were then cooled on ice and adjusted to pH 7.0 by the addition of 12 μl of 2 mM KH_2PO_4 . Each cell lysate was then added to 1.8 ml of TES buffer (10 mM TRIS-HCl, 100 mM NaCl, pH 7.0) containing 400 ng/ml of Hoechst dye (H 33258, Boehringer Mannheim, Germany). DNA was quantitated by measuring fluorescence on the Perkin-Elmer LS-5 fluorimeter (Excitation =330; Emission =455). Phenol extracted calf thymus DNA was used as a standard.

Reagents.

1,25(OH)₂-vitamin D₃ was a gift of Dr. Uskokovic (Hoffman-La Roche, Nutley, N.J.). Salmon Calcitonin (CT) was kindly provided by Dr. Orlowski (Rorer Central Research, Horsham, Penn.). Rat Calcitonin Gene-Related Peptide (CGRP) was purchased from Bachem, Inc. (Torrance, Calif.), and Forskolin from Calbiochem (La Jolla, Calif.). Recombinant Murine Interferon- γ (rMuIFN γ) (specific activity 6.8×10^6 units/mg) was prepared at Genentech, Inc. (South San Francisco, Calif.), stored at 4° C. in concentrated form and diluted immediately prior to use. The activity of rMuIFN γ was determined by a cytopathic effect inhibition assay using L929 murine fibroblasts challenged with encephalomyocarditis virus. This preparation contained 0.034 EU/mg by the limulus amoebocyte lysate test where 1 EU is the amount of limulus amoebocyte lysate-reactive material of US pharmacopeia reference standard endotoxin. The rabbit polyclonal antisera directed against rMuIFN γ (neutralization titer $=5 \times 10^4$ U/mg) was also prepared at Genentech, Inc.

Recombinant murine interleukin-1 β and a rabbit antibody directed against interleukin-1 β were provided by Dr. R. C.

Newton (Dupont, Glenolden, Penn.). A mAb directed against murine interleukin-1 α (mAb 161.1) was a gift of Dr. David Chaplin (Washington University School of Medicine, Washington, Mo.). IL-2, IL-6, anti-IL-6, recombinant human interleukin-2 and -6, and a polyclonal rabbit antiserum were purchased from Genzyme (Boston, Mass.). A polyclonal rabbit antiserum anti-CGRP was provided by Dr. Susan Amara.

Results

Giant Cell Formation in vitro:

The fusion of rat macrophages is easily induced in vitro by plating alveolar macrophages at maximal density, i.e., cell-cell contact, and culturing them for 3 to 4 days in medium supplemented with 5% human serum. When peritoneal macrophages from the same animals are grown in these conditions, they remain mononucleated and can therefore be used as a nonfusing control population. It was previously confirmed that giant cells differentiate in vitro by actual cell-cell fusion since DNA synthesis cannot be detected and that the total amount of DNA remains constant during culture in both alveolar and peritoneal macrophages. Therefore, the actual number of alveolar macrophages is decreased drastically by day 4 when each giant cell contains hundreds of nuclei. The important point is that the total number of nuclei remains constant so that we refer to giant cells and peritoneal macrophages as such or else as number of plated cells.

CGRP Inhibition of IL-1 Release

Inflammatory reactions are initiated in part by macrophages which secrete factors that activate lymphocytes in response to activation by bacterial infection and local stimuli. While IL-6 appears to be released by macrophages in the absence of stimulus, IL-1 secretion requires prior activation of the macrophages. Because of their apparent different regulatory mechanism and yet their critical role in immune reactions, these two cytokines were selected to investigate the putative role of calcitonin in multinucleated macrophage functional regulation.

Using a highly sensitive bioassay to measure IL-1 activity, detection of IL-1 activity was first attempted in the macrophage supernatants. IL-1 activity was very elevated (20 pg ± 5.0 per 10^6 plated cells, $n=3$) in the first 24-hour macrophages release media, i.e., following isolation and plating of the cells, most likely in response to mechanical stimuli and new environment. By day 3, both peritoneal and fused alveolar macrophage supernatants contained a 20-fold lower concentration of IL-1 activity. The addition of salmon calcitonin (sCT) to the release medium on day 3 reduced significantly the amount of IL-1 activity detected in giant cell supernatants but not in peritoneal macrophages. To verify the specificity of calcitonin effect, the macrophages from adjacent wells were treated with calcitonin gene-related peptide (CGRP), a neuropeptide encoded by the same gene as calcitonin. Surprisingly, CGRP prevented the release of IL-1 by both cell types. Because both CT and CGRP evoke a cAMP response in osteoclasts and muscle cells, respectively, whether this nucleotide was the second messenger involved in the regulation of IL-1 secretion was investigated. The addition of forskolin (a potent activator of adenylyl cyclase) to the release medium, was as effective as CGRP in blocking the secretion of IL-1 by both cell types. These biological activities were also blocked when the cell supernatants were preincubated with antibodies directed against IL-1 β and IL-1 α , thus demonstrating that IL-1 β and IL-1 α were responsible for the activity detected in this bioassay.

Simultaneously, both macrophage supernatants demonstrated significant IL-6 activity which level remained identical on day one and 3 (0.4 ± 0.1 fg per 10^6 plated cell per day). Of interest is the fact that neither sCT nor CGRP treatment of the cells modified this level.

Therefore, although both IL-1 and IL-6 appear to be constitutively released by macrophages cultured in these conditions, the regulation of their secretion is governed by different extra cellular factors. Of importance is the fact CGRP obviated IL-1 release from both cell types while CT effects were restricted to giant cells. These results suggest that peritoneal macrophages and giant cells differ functionally and that CT and CGRP may bind to different receptors.

Because $1,25(\text{OH})_2$ -vitamin D_3 has been shown to stimulate osteoclastic bone resorption and IL-1 release from macrophages, it was deemed critical to verify whether macrophages cultured in these conditions responded to these hormones and cytokine. As expected, the addition of $1,25(\text{OH})_2$ -vitamin D_3 stimulated the proliferation of CTLL cells, and probably IL-1 release but only by peritoneal macrophages. Therefore, these data not only confirm earlier reports on mononucleated macrophages but also demonstrate that giant cells fail to respond to $1,25(\text{OH})_2$ -vitamin D_3 , therefore providing further evidence that multinucleation modifies the immunological role played by macrophages.

To further investigate the molecular interactions controlling macrophage function, tests were next conducted to determine if CGRP could inhibit vitamin D_3 stimulated peritoneal macrophages. It was determined that, although to a lesser extent, CGRP was able to reduce significantly the IL-1 bioactivity present in peritoneal macrophage supernatants.

This inhibitory activity is not reversed by the immunoprecipitation of CGRP with an antibody anti-CGRP from the macrophage supernatants prior to testing in the IL-1 bioassay. Thus confirming that CGRP prevents directly IL-1 release by macrophages.

However, the addition of CGRP to IL-1 containing medium prevents the proliferative response of CTLL cytotoxic T cells in the IL-1 bioassay. This suggests that CGRP either inhibits IL-2 (and/or IL-4) production by proliferation. Because the treatment of the CTLL cells directly with CGRP, in the presence of IL-2, did not prevent significantly their proliferation, we conclude that CGRP prevents IL-2, and/or IL-4 and/or other cytokine secretion by LBRM-33 cells. Thus, CGRP suppresses the immune response at multiple levels by binding to macrophages and lymphocytes and modifying their function.

Taken together, these results suggest that giant cells release cytokines and that the neuropeptide CGRP directly prevents IL-1 and/or IL-2 (IL-4) release by giant cells, macrophages or lymphocytes. Moreover, the above results suggest that IL-1 secretion and/or IL-2/IL-4 inhibition is mediated by cAMP. To further investigate the mechanism by which CT and CGRP inhibits IL-1 secretion, their effect on cAMP production in peritoneal macrophages and giant cells was first investigated.

CGRP stimulates cAMP Production in Peritoneal Macrophages and Giant Cells but CT Effects are Restricted to Giant Cells

Both CT and CGRP evoke a cAMP response in osteoclasts and muscle cells, respectively. To detect a putative accumulation of cAMP in giant cells and peritoneal macrophages in response to sCT, peritoneal macrophages and giant cells were incubated for the indicated times in the absence or presence of either salmon calcitonin (sCT),

isobutylmethylxanthine (IBMX), or sCT plus IBMX. While sCT failed to induce a response in peritoneal macrophages at all time points examined, a pic of cAMP accumulation was detected as early as 5 minutes after addition of the peptide to fused alveolar macrophages. This lag time was reduced to 2 minutes when as little as $10 \mu\text{M}$ IBMX, a phosphodiesterase inhibitor and cAMP returned to basal level after 15 and 30 minutes, in the absence and presence of IBMX, respectively. Neither MEME alone or with IBMX were able to induce cAMP accumulation.

When the dose response effect of sCT on cAMP production in giant cells was examined, a dose-dependence of sCT was recorded. Half maximal stimulation of cAMP accumulation in giant cells (50 fmoles per 6.4×10^4 plated cells) was attained at a concentration of 2 nM. Therefore, giant cells but not peritoneal macrophages respond to sCT by elevating their concentration of cytoplasmic cAMP, thus corroborating the above results showing that CT inhibition IL-1 release is specific for giant cells. Conversely, when a dose-dependent cAMP accumulation was measured after 2 minutes of incubation with CGRP in the presence of IBMX, both mononucleated and fused macrophages responded. Although fused alveolar macrophages repeatedly accumulated more cAMP than peritoneal macrophages, the half maximal concentration of cAMP (300 vs. 80 fmoles per 5×10^4 plated cells, respectively) was also attained by the addition of 2 nM CGRP in both cell types.

To investigate whether this difference in amplitude was associated with multinucleation, cAMP accumulation in response to CGRP was recorded as a function of time. This difference was detected as early as 2 hours after plating the cells which both accumulated higher concentrations of cAMP with time in culture to reach a plateau by day 2 (fmoles per 5×10^4 cells). Medium alone or supplemented with IBMX was not able at any time point measured to induce a cAMP accumulation in these cells. Of importance is the fact that CGRP induced a larger accumulation of cAMP in macrophages than CT, thus correlating with their respective effects on IL-1-release inhibition. These data are consistent with the proposition that macrophages, whether mono- or multinucleated do express CGRP receptors and that culturing them increases the amplitude of their cAMP response to this neuropeptide. Moreover, these data strongly suggest that cAMP mediates the effects of both CT and CGRP on IL-1 secretion.

A human being suffering from chronic rheumatoid arthritis experiences an amelioration of the symptoms of the disease, e.g., pain and swelling of the affected joints, by the I.M. administration of 0.5 mg of CGRP every 24 hours until relief is noted. Similar results are observed by injection of the CGRP into an affected joint.

Osteoporosis has been recently linked to an abnormally high IL-1 release level. In accordance with this invention, CGRP is more potent than CT in inhibiting the degenerative process of the bone.

Organ transplant is accompanied by an acute inflammatory reaction associated with surgery and a chronic inflammatory reaction associated with graft rejection. In accordance with this invention, CGRP is useful in limiting the rejection process by reducing the inflammatory reaction.

IL-1 is secreted by keratinocytes (skin cells), endothelial cells (vascular wall), mesangial cells (kidney) and glial cells (brain); therefore, clinical applications for the use of CT and CGRP embrace a vast array of diseases involving all tissues and organs, since they are all vascularized, involved in a chronic or acute inflammatory reaction, e.g., in response to injury, surgery, infection or autoimmunity.

For example, CT and CGRP can be used to limit the inflammatory condition associated with surgery, with or without organ transplant, following cancer, trauma, metabolic diseases; orthopedic surgery, following or not trauma and associated with chronic inflammation and non-repair; allergic reactions involving skin (eczema), lung (asthma), eyes, digestive tract, nervous system, etc.

Additionally, because nervous tissue is rich in CGRP receptors and glial cells secrete IL-1, CGRP therefore may be used for treatment of mental problems, headaches and earaches; periodontal disease involves chronic inflammation, which leads to bone destruction and ultimately tooth loss. The use of CT or CGRP as a topical solution limits the course of this disease, as well as limiting gingivitis and periodontitis.

Because ovulation is associated with IL-1 release, inflammation and fever, CGRP can be used as an antipregnancy drug, e.g., as an oral contraceptive.

CGRP, as a potent immunosuppressor, can help facilitate pregnancy or feto-maternal biological interactions (prevent the rejection of the fetus in noncompatibility of blood types) and help to prevent rejection of transplanted/implanted organs.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

What is claimed is:

1. A method of down-regulating the immune response in a human being suffering from an inflammatory condition mediated by a hyperimmune response resulting from cytokine release and receptor expression by immune and non-immune cells, interleukin1 (IL-1) or IL-1 and interleukin-2 (IL-2) release from interleukin-1 and interleukin-2 releasing cells, which comprises administering thereto an immunosuppressive amount of calcitonin gene-related peptide (CGRP) effective to down-regulate the immune response and ameliorate the inflammatory condition.

2. A method according to claim 1, wherein the CGRP is administered by injection.

3. A method according to claim 2, wherein the injection is intramuscular.

4. A method according to claim 1, wherein the inflammatory condition is a chronic inflammatory disease.

5. A method according to claim 4, wherein the disease is rheumatoid arthritis.

6. A sterile pharmaceutical composition adapted for systemic administration by injection which comprises per unit dosage an IL-1 release inhibiting amount of CGRP from 10 to 100 mg per unit dosage, in admixture with a pharmaceutically acceptable carrier.

7. The method of claim 1, wherein an amount from 10 to 100 mg per unit dose of CGRT is administered thereto.

8. A method of treating diabetes which comprises administering to a human being suffering from the disease an immunosuppressive amount of calcitonin gene-related peptide (CGRP) effective to ameliorate the disease.

9. The method of claim 8, wherein an amount from 10 to 100 mg per unit dose of CGRP is administered thereto.

* * * * *

APPENDIX F
Dakhama Declaration

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

GELFAND et al.

Serial No.: 09/809,753

Filed: March 14, 2001

Atty. File No.: 2879-74

For: "METHOD FOR REDUCING
ALLERGEN-INDUCED AIRWAY
HYPERRESPONSIVENESS"

Group Art Unit: 1644

Examiner: Huynh, Phuong N.

Confirmation No.: 5001

DECLARATION OF
AZZEDDINE DAKHAMACommissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

I, Azzeddine Dakhama, declare as follows.

1. I am currently employed as an Associate Professor in the Department of Pediatrics, Division of Cell Biology at National Jewish Medical and Research Center in Denver, Colorado.
2. I am a co-inventor of the above-referenced patent application.
3. I have reviewed the pages of the documents attached as "Exhibit A." I was in the laboratory of Dr. Erwin Gelfand working as a Research Associate along with my colleague, Dr. Arikiko Kanehiro, when the work described in Exhibit A was performed. Furthermore, I confirm that I performed most of the work described in Exhibit A. Dr. Arikiko Kanehiro helped in a later part of the experiment by performing lung function measurements in my presence.
4. The header in bold on page 1 of Exhibit A includes "Azd/Ari." The letters "Azd" refer to me and "Ari" refers to Dr. Kanehiro's nickname. Pages 1 and 2 of Exhibit A are a

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printout from a computer file recording the experiment. I entered the information on pages 1 and 2 of Exhibit A into the computer. Page 3 of Exhibit A is an image of the computer information for that file. Page 3 shows that the file was created on August 25, 1999, and it was last modified on August 27, 1999. I labeled the experiment described on pages 1 and 2 of Exhibit A "Experiment 23JL99," which means that I initiated the experiment on July 23, 1999.

5. Pages 4 to 7 of Exhibit A are photocopies of handwritten notebook pages recorded by Dr. Kanehiro, which include the raw data of lung function measurements Dr. Kanehiro recorded for the experiment. I was with Dr. Kanehiro when he performed this part of the experiment. I typed the data he recorded at pages 4 to 7 of Exhibit A into the computer between August 25, 1999, and August 27, 1999, as shown on pages 1 and 2 of Exhibit A. Dr. Kanehiro dated the first two handwritten pages "8/25", meaning that he performed the measurements of lung function for this experiment on a first set of animals (numbered 1 to 7) and recorded the results on those pages on August 25, 1999. The third and fourth handwritten pages are dated "8/26", meaning that Dr. Kanehiro performed the measurements of lung function for this experiment on another set of animals (numbered 1 to 7) and recorded the results on those pages on August 26, 1999. I know that the dates are August 25 and 26, 1999, because the experiment is labeled "Experiment 23JL99," which meant that the experiment was initiated on July 23, 1999, and the sensitization and challenge of the mice, described on page 1 of Exhibit A, takes at least 30 days, placing the later part of the experiment that Dr. Kanehiro performed toward the end of August 1999. Moreover, the computer record of the creation of pages 1-2 of Exhibit A shows the creation date as August 25, 1999, with the date last modified as August 27, 1999, one day after the conclusion of the experiment as recorded in Dr. Kanehiro's handwriting on pages 6-7 of Exhibit A. Dr. Kanehiro also wrote "with Azd" on each of the first pages for

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August 25 (page 4) and August 26 (page 6), further confirming that this was the experiment with which Dr. Kanehiro assisted me and which correlates with pages 1-2 of Exhibit A. Finally, the data that I typed onto pages 1-2 are clearly the same data that Dr. Kanehiro recorded by hand on August 25-26. While Dr. Kanehiro was conducting his part of the experiment, I was in the same room preparing the mouse lungs for further evaluation after Dr. Kanehiro's part of the experiment was completed. Once Dr. Kanehiro was finished with a mouse, he would remove the mouse from the chamber of the physiology equipment and hand it to me for the further evaluation.

6. By August 1999, I was very familiar with the purpose of the experiment, and the experimental protocol, described in Exhibit A. I also consider that I was very skilled in the art in this field at that time. As noted in the "Objective" and "Hypothesis" on page 1 of Exhibit A, the purpose of the experiment was to study whether calcitonin gene related peptide (CGRP) would inhibit methacholine (MCh) induced airway hyperresponsiveness (AHR) in mice sensitized and exposed to the allergen ovalbumin (OVA), which was a mouse model of asthma. The abbreviations (CGRP), (MCh), (AHR), and (OVA) were commonly used in Dr. Gelfand's laboratory and by scientists at that time as shorthand for the terms calcitonin gene related peptide, methacholine, airway hyperresponsiveness, and ovalbumin, respectively.

7. This mouse model was used extensively in Dr. Gelfand's laboratory in 1999, and was known by those skilled in this field as an acceptable model for testing allergen-induced AHR. The following two exemplary articles discuss this mouse model: Hamelmann et al., "Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography," *Am. Respir. Crit. Care*, 156:766-775 (1997) (attached as Exhibit B); and Takeda et al., "Development of Eosinophilic Airway Inflammation and Airway

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Hyperresponsiveness in Mast Cell-deficient Mice, *J. Exp. Med.*, 186:449-454 (1997) (attached as Exhibit C). Those documents describe the mouse model in detail and provide experimental data showing that the model is a valid model for allergen-induced AIR, which models AIR *in vivo* in humans suffering from bronchial asthma. Hamelmann et al. and Takeda et al. show that the mouse model shares many characteristics with human respiratory conditions associated with allergic inflammation, including an IgE-associated immune response, a dependence on a Th2-type response, an eosinophil response, and both a marked and evolving hyperresponsiveness of the airways. See Hamelmann et al. at page 766, paragraph bridging the first and second columns; at page 770, Tables 1 and 2; at page 770, Figure 4; at page 773, Fig. 8; and at page 774, second column, last paragraph; and Takeda et al. at page 450, Figures 1 and 2; at page 450, Figure 4; and at page 452, first column, first full paragraph.

8. The mouse model uses ovalbumin as an experimental allergen. Ovalbumin was commonly referred to by the Gelfand laboratory and by the art in general as "OVA" or "Ova." Hamelmann et al. and Takeda et al., confirm this abbreviation. In an experiment using this mouse model, the mice are first "sensitized" (made sensitive or reactive) to the allergen (OVA) by repeated, systemic (intraperitoneal injection, abbreviated "i.p." or "ip") exposure to the OVA allergen. See Hamelmann et al. at page 767 "Sensitization and Airway Challenge", and Takeda et al. at page 449 "Sensitization and Airway Challenge". Exhibit A discusses this sensitization in the experiment performed by me as follows: "Balb/c mice were sensitized to OVA (2 i.p.) on day 0 and day 14." See Exhibit A at page 1.

9. This systemic sensitization with OVA is then followed by repeated airway challenge with the same allergen (OVA) in aerosol form (*e.g.*, nebulized administration, which was referred to by the Gelfand laboratory in shorthand as "N" or "Neb"). See Hamelmann et al.

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at page 767 "Sensitization and Airway Challenge", and Takeda et al. at page 449 "Sensitization and Airway Challenge". Exhibit A discusses this challenge with OVA in the experiment performed by me as follows: "On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." See Exhibit A at page 1.

10. Subsequent to the aerosol challenge with OVA, the mice are subjected to increasing doses of aerosolized methacholine (MCh). See Hamelmann et al. at page 768, col. 2, first full paragraph, and Takeda et al. at paragraphs spanning pages 449-450 under "Determination of Airway Responsiveness"; and at page 452, Figure 4. It was known that in such a model, after exposure to the provoking agent MCh, mice that have been sensitized to OVA and subsequently challenged with OVA show increased AIIR compared to control mice that have not been both sensitized and challenged with the allergen OVA. See Takeda et al. at page 451, second column, second paragraph, and page 452, second column, lines 3-7 of second paragraph. Thus, it is clear that in the model the AIIR is allergen-induced.

11. In 1999, there were different acceptable methods for measuring AHR. Hamelmann et al. discusses a noninvasive method, and Takeda et al. discusses an invasive method. Dr. Kanehiro was very experienced in the invasive method, which he used in the work described in Exhibit A. After I sensitized and challenged the mice with OVA as described on page 1 of Exhibit A, I gave the mice to Dr. Kanehiro. In August 1999, Dr. Kanehiro practiced an invasive method for measuring AHR in the mouse model similar to the method described in Takeda et al. at page 449, second column, to page 450, first column. Dr. Kanehiro used that method in the work described in Exhibit A. In that method, Dr. Kanehiro anesthetized and tracheostomized the mice. Dr. Kanehiro then attached a connector to the tracheostomy tube, with ports connected to the inspiratory and expiratory sides of two ventilators. One ventilator

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was used for mechanical ventilation of the lung and the other was used to administer the MCh into the lung. (In the method discussed in Takeda et al., only one ventilator was used.) Dr. Kanehiro connected another port of the connector to a pressure transducer, which was in turn connected to a plethysmograph.

12. The mice were then challenged with increasing doses of MCh. See Takeda et al. at paragraphs spanning pages 449-450 under "Determination of Airway Responsiveness"; and at page 452, Figure 4. After each dose of MCh, transpulmonary pressure was detected with the pressure transducer. *Id.* at page 450, first column. Also, "[c]hanges in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer" *Id.* Also, "[f]low was measured by digital differentiation of the volume signal." *Id.* "Lung resistance (R_L) and dynamic compliance (C_{dyn}) were continuously computed . . . by fitting flow, volume, and pressure to an equation of motion." *Id.* "Maximum values of R_L and minimum values of C_{dyn} were taken to express changes in murine airway function." *Id.* As discussed above, this is the method Dr. Kanehiro used in the work described in Exhibit A. The increasing doses that Dr. Kanehiro used in the work described in Exhibit A are shown in the left hand column on pages 4 to 7 of Exhibit A as "1.56," "3," "6.25," and "12.5." Those doses are also shown in left hand column of the copy of the data at pages 1 and 2 of Exhibit A.

13. In general, one skilled in the relevant art in August 1999 understood that an increase in R_L reflects both narrowing of the conducting airways and alterations in the lung periphery, while decreases in C_{dyn} reflect events in the lung periphery (Reviewed in Irvin and Bates, *Respiratory Research*, 4:1-9 (2003) (attached as Exhibit D), at page 5, col. 2). Increases

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in R_I , and decreases in C_{dyn} correlate with increased AHR. See Takeda et al., at page 451, last four lines of col. 2; Figure 4.

14. We used this mouse model in Dr. Gelfand's laboratory to test for the effect of treatment with a test agent on AHR. Mice that were sensitized and challenged with OVA, but which did not receive the experimental treatment, served as a positive control. A negative control for the experiment was generated by exposing one group of mice to PBS (a buffer) instead of OVA during the sensitization and nebulization challenge period. Alternatively, negative control mice could be exposed to PBS during the sensitization period and then exposed to the nebulized OVA during the challenge period, or vice versa (OVA sensitization and PBS challenge). AHR was then measured in the mice after inducing airway responses (bronchoconstriction) by exposing the mice to a provoking agent (stimulus), such as MCh. Negative control mice would not show significant AHR in response to the provoking stimulus MCh. The positive control mice that have been both sensitized to, and challenged with, OVA would respond to MCh exposure with a significant AHR compared to the negative control mice, both in terms of the dose of MCh required to increase AHR (a lower dose will result in more AHR), and in the elevation of the maximal response to MCh.

15. By comparing the AHR of the treated mice to the AHR of the positive and negative control mice, we could determine if administration of the test agent resulted in inhibition of allergen-induced AHR compared to mice that did not receive treatment with the test agent. In August 1999, this was an accepted method of testing whether a test agent inhibited allergen-induced AHR in mammals compared to mammals that did not receive treatment with the test agent.

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16. Exhibit A shows the use of this method to test whether administration of the test agent CGRP would inhibit allergen-induced AHR in the mice sensitized to, and challenged with, OVA, compared to mice that were not administered CGRP. Exhibit A describes four groups of mice that I prepared.

17. The first group had four mice that I prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. The mice in the first group were then pretreated with an i.p. injection of CGRP (200 μ l of 10^{-6} M) two hours prior to MCh exposure. Exhibit A at page 1. The results Dr. Kanchiro obtained with the mice in this first group are shown at pages 4 and 5 of Exhibit A with the term "IPN + CGRP," mice (1), (2), (3), and (4). I typed in that same data into the computer as shown on page 1 of Exhibit A under the heading "Group 1: OVA ip/Neb + CGRP." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, Dr. Kanchiro used the abbreviation "IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. As shown at page 1 of Exhibit A, I used the term "OVA ip/Neb" for the same mice. The "+ CGRP" in the terms at pages 1 and 4 indicates that the mice were also administered CGRP.

18. The second group had three mice that I prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. The mice in the second group were then pretreated with an "i.p. injection of CGRP antagonist (100 μ l of 10^{-5} M) followed by injection of CGRP (200 μ l of 10^{-6} M)" two hours prior to MCh exposure. Exhibit A

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at page 1. The results Dr. Kanehiro obtained with the mice in this second group are shown at page 5 of Exhibit A with the term "IPN + α -mAb + CGRP," mice (5), (6), and (7). I typed in that same data into the computer as shown on page 1 of Exhibit A under the heading "Group 2: OVA ip/Neb + Antag + CGRP." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, Dr. Kanehiro used the abbreviation "IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. As shown at page 1 of Exhibit A, I used the term "OVA ip/Neb" for the same mice. The terms "+ α -mAb" and "+ Antag" refer to the CGRP antagonist. The "+ CGRP" in the terms at pages 1 and 4 indicates that the mice were also administered CGRP.

19. The third group had three mice that I prepared as follows. The mice "were not sensitized and not challenged [with OVA]." These were negative control mice which also were not administered CGRP. Exhibit A at page 1. The results Dr. Kanehiro obtained with the mice in this third group are shown at page 6 of Exhibit A with the term "Saline Neb", mice (1), (2), and (3). I typed in that same data into the computer as shown on page 1 of Exhibit A under the heading "Group 3: PBS ip/Neb untreated (-CTL)." The terms "Saline Neb" and "PBS ip/Neb" indicates that the mice were neither sensitized to, nor challenged with, OVA. The term that Dr. Kanehiro used "Saline Neb" did not include the term "+ CGRP," which indicates that the mice were not administered CGRP. This same information is conveyed in the Group 3 designation that I used at page 1 of Exhibit A with the term "untreated (-CTL)."

20. The fourth group had three mice that I prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. These mice were

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positive control mice which were not administered CGRP. Exhibit A at page 1. The results Dr. Kanchiro obtained with the mice in this fourth group are shown at page 7 of Exhibit A with the term "OVA IPN," mice (4), (5), (6), and (7). I typed in that same data into the computer as shown on page 1 of Exhibit A under the heading "Group 4: OVA ip/Neb untreated (-CTL)." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, Dr. Kanchiro used the abbreviation "OVA IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. As shown at page 1 of Exhibit A, I used the term "OVA ip/Neb" for the same mice. The term that Dr. Kanchiro used "OVA IPN" did not include the term "+ CGRP," which indicates that the mice were not administered CGRP. This same information is conveyed in the Group 4 designation that I used at page 1 of Exhibit A with the term "untreated (+CTL)."

21. I provided the mice from Groups 1 to 4 to Dr. Kanchiro so that he could expose the mice to the MCh and make the measurements of lung resistance (R_L) and dynamic compliance (C_{dyn}) as discussed above. I now discuss Dr. Kanchiro's handwritten data on pages 4-7 of Exhibit A, which I copied into the tables on pages 1-2 of Exhibit A. For each mouse, Dr. Kanchiro recorded the body weight of the mouse (BW). Dr. Kanchiro then recorded for each mouse, two values for each of a baseline (BL) (prior to administration of MCh), saline administration (SAL) (0 mg/ml MCh), and then doubling doses of MCh from 1.56 mg/ml to 12.5 mg/ml. Referring to the two values recorded at each data point, the first number is the value for R_L and the second number is the value for C_{dyn} . The number that Dr. Kanchiro recorded for the C_{dyn} is written in shorthand. The actual number was 10^{-3} of the recorded number.

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22. On August 25, 1999 (8/25), as shown on pages 4-5 of Exhibit A, we evaluated the first seven mice, and on August 26, 1999 (8/26), as shown on pages 6-7 of Exhibit A, we evaluated the last seven mice. The evaluations had to be split over two days because evaluation of 14 mice would have required too much time for one day. As shown on page 4, Dr. Kanehiro evaluated the first group of mice discussed above, which Dr. Kanehiro designated "IPN + CGRP." As discussed above, that was Dr. Kanehiro's shorthand for referring to "OVA ipNeb + CGRP" mice (allergen-sensitized and challenged) that also received the CGRP. There were four mice in that group, designated by numbers. Dr. Kanehiro also evaluated the three mice in the second group of mice discussed above (CGRP + CGRP antagonist group) as shown on page 5, which Dr. Kanehiro referred to as "IPN + α -mAB + CGRP," corresponding to "OVA ipNeb + Antag + CGRP" mice (allergen-sensitized and challenged) that also received the CGRP and CGRP antagonist. As shown on page 6, Dr. Kanehiro evaluated the three mice in the third group of mice discussed above (the negative control group), which Dr. Kanehiro designated as "Saline Neb," corresponding to "PBS ip/Neb untreated (-CTL)" on page 1 of Exhibit A. As shown on page 7, Dr. Kanehiro evaluated the four mice of the fourth group of mice discussed above (the positive control group), which Dr. Kanehiro designated "OVA IPN," corresponding to "OVA ip/Neb untreated (+CTL)" on pages 1-2 of Exhibit A. Mouse 6 on page 5 and Mouse 7 on page 8 both died during the procedure, which can happen. This explains the lack of data and a handwritten line in these columns.

23. These data were evaluated by comparing the changes in R_L in response to increasing doses of MCh, represented by the first number recorded for each mouse, in the presence and absence of CGRP. Changes in C_{dyn} were also evaluated by comparing the change in this number in response to increasing doses of MCh, represented by the second number

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recorded for each mouse, in the presence and absence of CGRP. Visual inspection of the raw data showed that if the positive and negative control mice are compared, the R_L values for the positive control mice appeared to be significantly higher in response to increasing doses of MCh as compared to the R_L values for the negative control mice. Compare, for example, the R_L values for mouse 1 in Group 3 (mouse 1 in the 8/26 data) to mouse 1 in Group 4 (mouse 4 in the 8/26 data). With respect to C_{dyn} , C_{dyn} values for the positive control mice appeared to be significantly lower in response to increasing doses of MCh as compared to the C_{dyn} values for the negative control mice, which was expected. With regard to the CGRP treated mice (Group I), viewing, for example, the R_L values for mouse 1 of Group 1 (mouse 1 in the 8/25 data), these values appeared to be more similar to the negative control mice than the positive control mice, as did the C_{dyn} values, indicating that CGRP inhibited AIIR in the mice. In contrast, viewing, for example, the R_L values for mouse 5 of Group 2 (mouse 5 in the 8/25 data), which represented CGRP antagonist mice, these values appeared to be more similar to the positive control mice than the negative control mice, as did the C_{dyn} values, indicating that the inhibitory effects of CGRP were abolished in the presence of a CGRP antagonist. Therefore, I concluded from looking at the raw data for this experiment that CGRP inhibited AIIR in allergen-sensitized and challenged mice as compared to in the absence of CGRP, and furthermore, that the effects were directly due to the CGRP, since the CGRP antagonist reversed that result.

24. I confirm that the experiment described in Exhibit A was completed on August 26, 1999. I also confirm that on that day, I recognized that the data showed that the administration of CGRP to allergen-sensitized and challenged mice inhibited allergen-induced airway hyperresponsiveness (AIIR) in the mice, as compared to in the absence of administration of CGRP. I also confirm that on that day, I recognized that the data from that experiment

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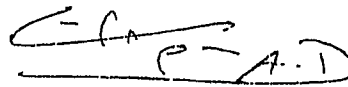
demonstrated that administration of CGRP to a mouse that has allergen-induced AIIR inhibited allergen-induced AIIR in the mouse as compared to in the absence of administration of CGRP.

It is my opinion that anyone skilled in the relevant field reading these data would have arrived at the same conclusion on August 26, 1999.

25. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

02-22-2008

Date



Azzedine Dakhama

EXHIBIT A

Experiment 23JL99

Azd/Ari

Objective: to study the effect of CGRP on MCh-induced AHR in mouse model of asthma

Hypothesis: CGRP inhibits MCh-induced AHR in mice sensitized and exposed to OVA

Balb/c mice were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days. Control animals were not sensitized and not challenged. At 2 h prior to MCh responsiveness, animals pretreated either with i.p. injection of CGRP (200 μ l of 10^{-6} M) or i.p. injection of CGRP antagonist (100 μ l of 10^{-5} M) followed by injection of CGRP (200 μ l of 10^{-6} M). After measurement of MCh-induced AHR, the lungs were inflated with OCT:PFA and embedded in OCT for histology.

RESULTS (AHR)

Group 1: OVA ip/Neb + CGRP

	1	2	3	4
BL	0.58/68	0.73/62	0.47/72	0.63/62
SAL	0.79/39	0.91/50	0.65/48	0.76/50
1.56	0.84/34	1.01/48	0.70/31	0.84/46
3.12	0.94/29	1.16/38	0.91/28	1.14/25
6.25	1.36/24	1.70/22	1.25/21	1.46/21
12.5	1.32/23	2.14/16	1.72/12	2.35/12

Group 2: OVA ip/Neb + Antag + CGRP

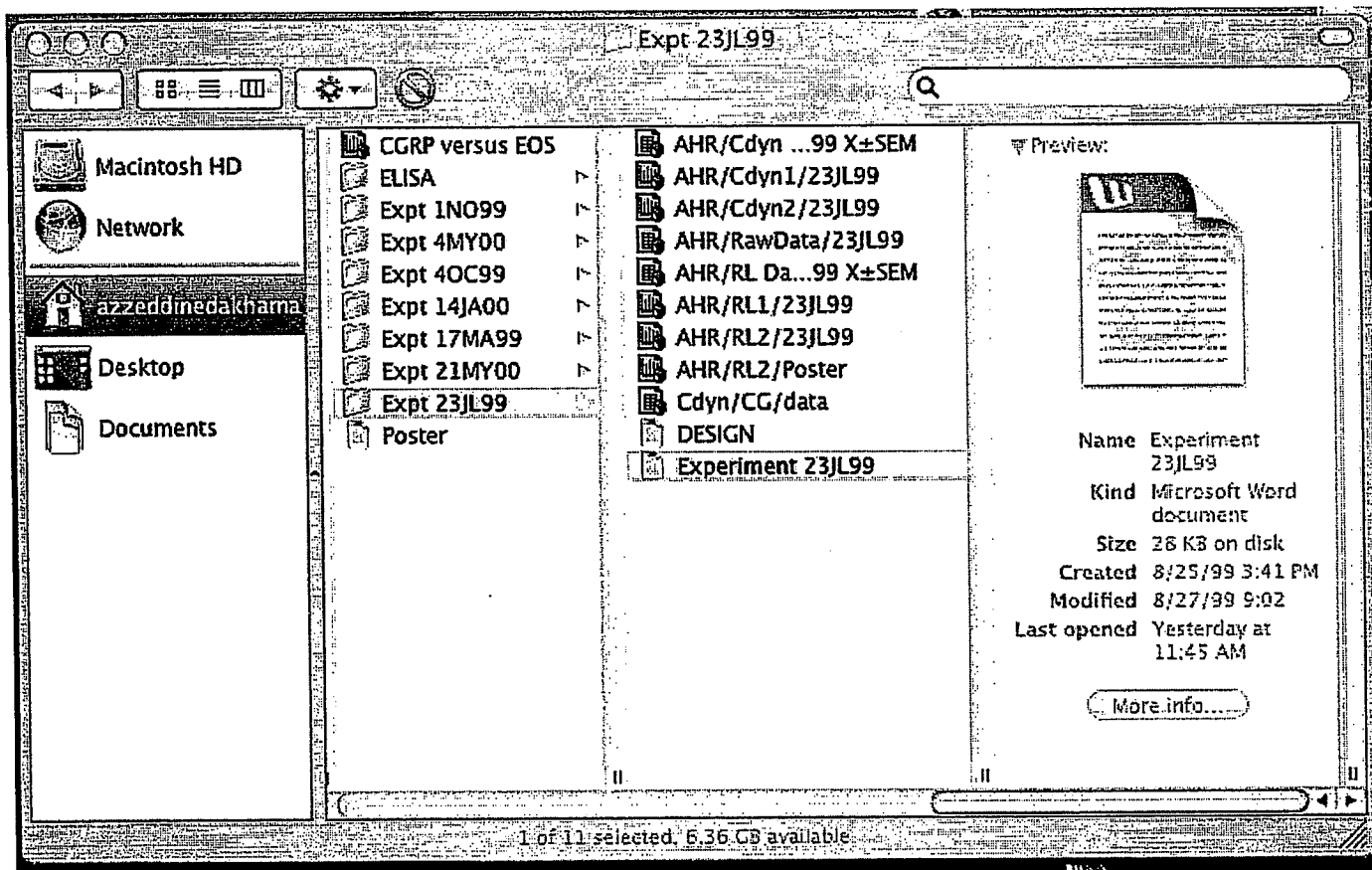
	5	6	7
BL	0.55/61	Collapsed	0.51/61
SAL	0.75/35		0.65/42
1.56	1.05/25		0.77/37
3.12	1.56/21		1.94/13
6.25	2.58/13		4.15/6
12.5	4.02/6		4.36/5

Group 3: PBS ip/Neb untreated (-CTL)

	1	2	3
BL	0.59/63	0.50/59	0.47/60
SAL	0.70/60	0.53/57	0.49/56
1.56	0.81/58	0.64/45	0.60/47
3.12	0.95/38	1.09/31	0.92/38
6.25	1.25/27	1.35/26	1.70/19
12.5	1.40/25	1.75/15	1.48/20

Group 4: OVA ip/Neb untreated (+CTL)

	1	2	3
BL	0.64/64	0.57/72	0.60/66
SAL	0.76/41	0.66/60	0.71/47
1.56	1.07/34	0.90/37	0.96/33
3.12	1.64/21	2.00/8	1.78/16
6.25	2.87/11	3.45/8	3.33/8
12.5	5.03/7	4.20/4	4.25/5



Expt 23 JUL 99

8/25 - ①

with AZP

① 1 PM
+ CRP

②

③

BW

28

22.2

27

BL 0.58/68

0.73/62

0.47/72

sal 0.79/39

0.91/50

0.65/48

1.56 0.84/34

1.01/48

0.70/31

3 0.94/29

1.16/38

0.91/28

6.25 1.36/24

1.70/22

1.25/21

12.5 ~~1.17/20~~

1.32/23

2.14/16

1.72/12

8/28 - ②

④

h

⑤

1PN
+ 2-MAD
+ C412P

⑥

q

⑦

q

BW

28

26.5

33.5

30

BL 0.63/62

0.55/61

0.57/61

sol 0.76/50

0.75/35

0.65/42

1.56 0.84/46

1.05/28

0.77/37

3 1.14/28

1.56/21

1.94/13

6.25 1.46/21

2.58/13

4.15/6

12.5 2.35/12

4.02/6

4.36/5

8/26 ①

with AZD

①

②

③

Salin Nap.

BC

WJ

J/

Jo. f

BC 0.59/63

0.50/59

0.47/60

sal 0.70/60

0.53/57

0.48/56

1.58 0.81/58

0.64/45

0.60/47

0.129 0.95/38

1.09/31

0.92/38

6.28 1.25/27

1.35/26

1.70/19

12.5 1.40/25

1.75/15

1.48/20

26 (2)

(4) 001 / PN (5)

(6)

(7)

BD

27

WN

26

BL 0.64/64 0.57/72 0.60/66

sal 0.76/41 0.66/60 0.71/47

sf 1.07/84 0.90/87 0.96/88

w 1.64/24 2.00/8 1.78/16

6 2.87/11 w. 45/8 w. 25/8

12 5.03/7 4.20/4 4.25/5

EXHIBIT B

Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography

E. HAMELMANN, J. SCHWARZE, K. TAKEDA, A. OSHIBA, G. L. LARSEN, C. G. IRVIN,
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To study the mechanisms and kinetics underlying the development of increased airway responsiveness (AR) after allergic sensitization, animal models have been invaluable. Using barometric whole-body plethysmography and increases in enhanced pause (Penh) as an index of airway obstruction, we measured responses to inhaled methacholine in conscious, unrestrained mice after sensitization and airway challenge with ovalbumin (OVA). Sensitized and challenged animals had significantly increased AR to aerosolized methacholine compared with control animals. AR measured as Penh was associated with increased IgE production and eosinophil lung infiltration. In a separate approach we confirmed the involvement of the lower airways in the response to aerosolized methacholine using tracheotomized mice. Increases in Penh values after methacholine challenge were also correlated with increased intrapleural pressure, measured via an esophageal tube. Lastly, mice demonstrating AR using a noninvasive technique also demonstrated increased pulmonary resistance responses to aerosolized methacholine when measured using an invasive technique the following day in the same animals. The increases in Penh values were inhibited by pretreatment of the mice with a β_2 -agonist. These data indicate that measurement of AR to inhaled methacholine by barometric whole-body plethysmography is a valid indicator of airway hyperresponsiveness after allergic sensitization in mice. The measurement of AR in unrestrained, conscious animals provides new opportunities to evaluate the mechanisms and kinetics underlying the development and maintenance of airway hyperresponsiveness and to assess various therapeutic interventions. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *AM J RESPIR CRIT CARE MED* 1997;156:766-775.

Airway hyperresponsiveness (AHR), airway inflammation, and reversible airway obstruction are the hallmarks of bronchial asthma (1). In the development of AHR, neurogenic abnormalities (2) and airway inflammation (3), characterized by eosinophil infiltration (4), and the release of inflammatory mediators and cytokines (5) have been implicated. Animal models have been developed to investigate the pathogenetic mechanisms involved in the development of AHR, providing a means for *in vivo* manipulation and *in vitro* study of easily accessible cells and tissue (6). These models also permit the testing of different protocols and reagents for the prevention of AHR and airway inflammation, approaches that are unsuitable or impossible to perform in patients. Because of the advanced

understanding of the immune system in mice and the availability of reagents and genetically altered mice, murine models of AHR have become increasingly important in defining which cells and factors are involved (7). Several reports have described the roles that interleukin-4 (IL-4) (8), IL-5 (9, 10), and eosinophil lung infiltration (11, 12) play in the development of AHR in allergen-sensitized mice, but additional studies are needed to better define the kinetics and mechanisms underlying AHR.

To date, three different approaches have been used to measure altered airway function in mice: *in vitro* measurement of tracheal smooth muscle contractility after electrical field stimulation (13), *in vivo* measurement of lung resistance or compliance after intravenous injection of bronchoconstrictive agents such as methacholine and serotonin (14, 15), and *in vivo* measurement of peak airway opening pressure (16). Each of these methods have their limitations. The *in vitro* technique correlates well with allergic airway sensitization (13) and appears to reflect increased acetylcholine release caused by M_2 receptor dysfunction in sensitized animals (17). However, the influence of mucus production, mucosal edema, or other changes in the lower airways after allergic sensitization are not reflected in monitoring airway responsiveness with this technique. The *in vivo* techniques (14-16) perform measurements of AHR in tracheotomized and ventilated animals. The influence of anes-

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thetia and of the operating procedures on the readings is not well defined. Furthermore, intravenous challenge of the mice with bronchoconstrictive agents might not solely reflect physiologic stimulation of airway smooth muscles. Finally, this method is technically demanding and time-consuming.

In this report, we present data from studies carried out using barometric whole-body plethysmography (WBP) for the measurements of AR in unrestrained and conscious mice after sensitization and airway challenge with allergen. WBP has several potential advantages when compared with the above-mentioned techniques: it is technically not as demanding, allows measurements of AR to aerosolized stimulants, and provides a technique for repeated and long-term measurements of AR, as killing of the mice after the measurements is not required, thus allowing the evaluation of kinetics and treatment protocols of AHR. However, because of the indirect and noninvasive measurement of airway function, thorough evaluation of WBP is necessary before it can be accepted as a technique to measure AHR. The influence of upper airway responsiveness and changes in breathing pattern (respiratory rate, tidal volume) on the read-out of WBP need to be evaluated. We addressed these problems by measuring AR by WBP in tracheotomized animals, simultaneously measuring WBP and intrapleural pressure, and sequentially measuring WBP and lung resistance in the same animals. Further, we studied the effects of changes in the respiratory rate and of the response to a bronchodilator on WBP. The data shown in this study indicate that WBP in mice provides a valid assessment of AHR in allergen-sensitized mice.

METHODS

Animals

Female BALB/c mice 8 to 12 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Center for Immunology and Respiratory Medicine.

Sensitization and Airway Challenge

Groups of mice (three to four mice/group/experiment) receiving the following treatment were studied: (1) no treatment (N); (2) sensitization to OVA plus airway challenge with PBS (ip); (3) sham-sensitization with PBS plus airway challenge with OVA (Neb); (4) sensitization plus challenge with OVA (ipNeb). Mice were sensitized by intraperitoneal injection of 20 μ g OVA (Sigma, St. Louis, MO) emulsified in 2 mg aluminum hydroxide (AlumInject; Pierce Chemical, Rockford, IL) in a total volume of 100 μ l on Days 1 and 14. Mice were challenged via the airways with OVA (1% in PBS) or PBS for 20 min on Days 28, 29, and 30 by ultrasonic nebulization and assessed on Day 31 for AR. In selected mice, invasive methods to measure pulmonary resistance were employed on Day 32.

Determination of Airway Responsiveness

AR was measured in unrestrained animals by barometric plethysmography (18) using whole body plethysmography (WBP) (Figure 1) (Buxco, Troy, NY). Before taking readings, the box was calibrated with a rapid injection of 150 μ l air into the main chamber. Measured were pressure differences between the main chamber of the WBP containing the animal, and a reference chamber (box pressure signal). This box pressure signal is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. A pneumotachograph with defined resistance in the wall of the main chamber acts as a low-pass filter and allows thermal compensation (Figure 1). The time constant of the box was determined to be approximately 0.02 s.

Inspiration and expiration are recorded by establishing start-inspiration and end-inspiration as the box pressure/time curve crosses the

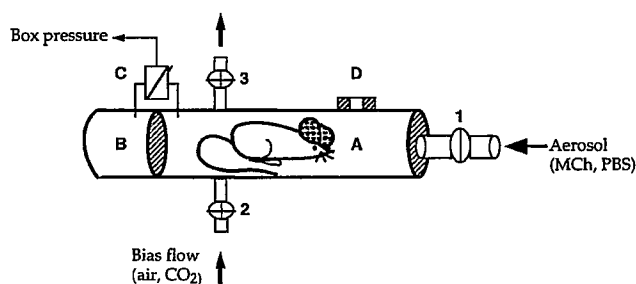


Figure 1. Schematic diagram of the whole-body plethysmograph. (A) Main chamber containing the mouse. (B) Reference chamber. (C) Pressure transducer connected to analyzer. (D) Pneumotachograph. (1) Main inlet for aerosol closed by valve. (2) Inlet for bias flow with four-way stopcock; (3) Outlet for aerosol with four-way stopcock.

zero point (see Figure 2). Start of an inspiration is determined by extrapolating from a straight line drawn from two levels of the rising inspiratory phase of the box pressure signal. Time of inspiration (T_i) is defined as the time from the start of inspiration to the end of inspiration; time of expiration (T_e) as the time from the end of inspiration to the start of the next inspiration (Figure 2). The maximum box pressure signal occurring during one breath in a negative or positive direction is defined as peak inspiratory pressure (PIP) or peak expiratory pressure (PEP), respectively (Figure 2). Recordings of every 10 breaths are extrapolated to define the respiratory rate in breaths per minute. The relaxation time (T_r) is defined as the time of pressure decay to 36% of the total expiratory pressure signal (area under the box pressure signal in expiration). This may thus serve as a correlate to the time constant (RC) of the decay of the volume signal to 36% of the peak volume in passive expiration. During bronchoconstriction, the main alteration in the signal occurs during early expiration and leads to changes in the waveform of the box pressure signal (19, 20). This

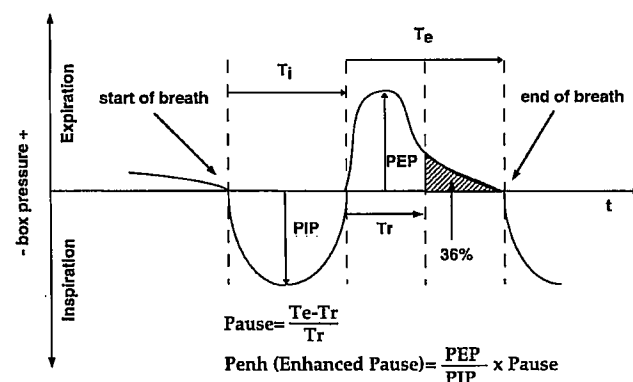


Figure 2. Computation of the parameters measured by barometric plethysmography. Schematic figure of a box pressure wave in inspiration (down) and expiration (up) explaining the computation of the parameters measured by WBP. T_i = inspiratory time (s), time from start of inspiration to end of inspiration; T_e = expiratory time (s), time from end of inspiration to start of next inspiration; PIP = peak inspiratory pressure (ml/s), maximal negative box pressure occurring in one breath; PEP = peak expiratory pressure (ml/s), maximal positive box pressure occurring in one breath; f = frequency (breaths/min), respiratory rate; T_r = relaxation time (s), time of the pressure decay to 36% of total box pressure during expiration.

change in the waveform can be quantified comparing the mean expiratory box pressure during early expiration (MP1) with the mean expiratory box pressure during late expiration (MP2) by measurement of Pause (Figure 2) where MP1 = mean expiratory box pressure 1; MP2 = mean expiratory box pressure 2; P = expiratory box pressure:

$$\begin{aligned} \text{MP1} &= \frac{0.65 P}{\text{Tr}} \\ \text{MP2} &= \frac{0.35 P}{\text{Te} - \text{Tr}} \\ \text{Pause} &= \frac{\text{Te} - \text{Tr}}{\text{Tr}} = \frac{0.35 P}{0.65 P} \times \frac{\text{MP1}}{\text{MP2}} - \frac{\text{MP1}}{\text{MP2}} \end{aligned}$$

During bronchoconstriction, the changes in box pressure during expiration (PEP) are more pronounced than during inspiration (PIP) (20) (see Figure 3). This is reflected by the formula for enhanced pause (Penh), a dimensionless value used in this study to empirically monitor airway function:

$$\text{Penh} = \text{Pause} \times \frac{\text{PEP}}{\text{PIP}}$$

Penh reflects changes in the waveform of the box pressure signal from both inspiration and expiration (PIP, PEP) and combines it with the timing comparison of early and late expiration (Pause). Penh is not a function of the absolute box pressure amplitude or the respiratory rate, but rather a junction of the proportion of the pressure signal from

inspiration and expiration and of the timing of expiration. An example of the box pressure waveform from a normal mouse before and after challenge with aerosolized methacholine is shown in Figure 3, demonstrating the changes in the waveform as well as in Pause and Penh after agonist inhalation.

Mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized PBS or methacholine in increasing concentrations (3 to 50 mg/ml) were nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. Airway reactivity was expressed as a fold increase for each concentration of MCh (Penh_{MCh}) compared with Penh values after PBS challenge (Penh_{PBS}).

For the quantification of the dose-response to methacholine, the linear regression of Penh on log base 2 was calculated for individual mice. The log dose corresponding to an increase in Penh of 100 or 200%, respectively, was determined, and the average log doses of the different groups were compared by analysis of variance. The data are reported as the geometric mean with the lower and upper limit of 95% confidence interval.

Studies with Mechanical Ventilation

In order to determine the influence of breathing frequency and tidal volume as well as bronchoconstriction under controlled conditions,

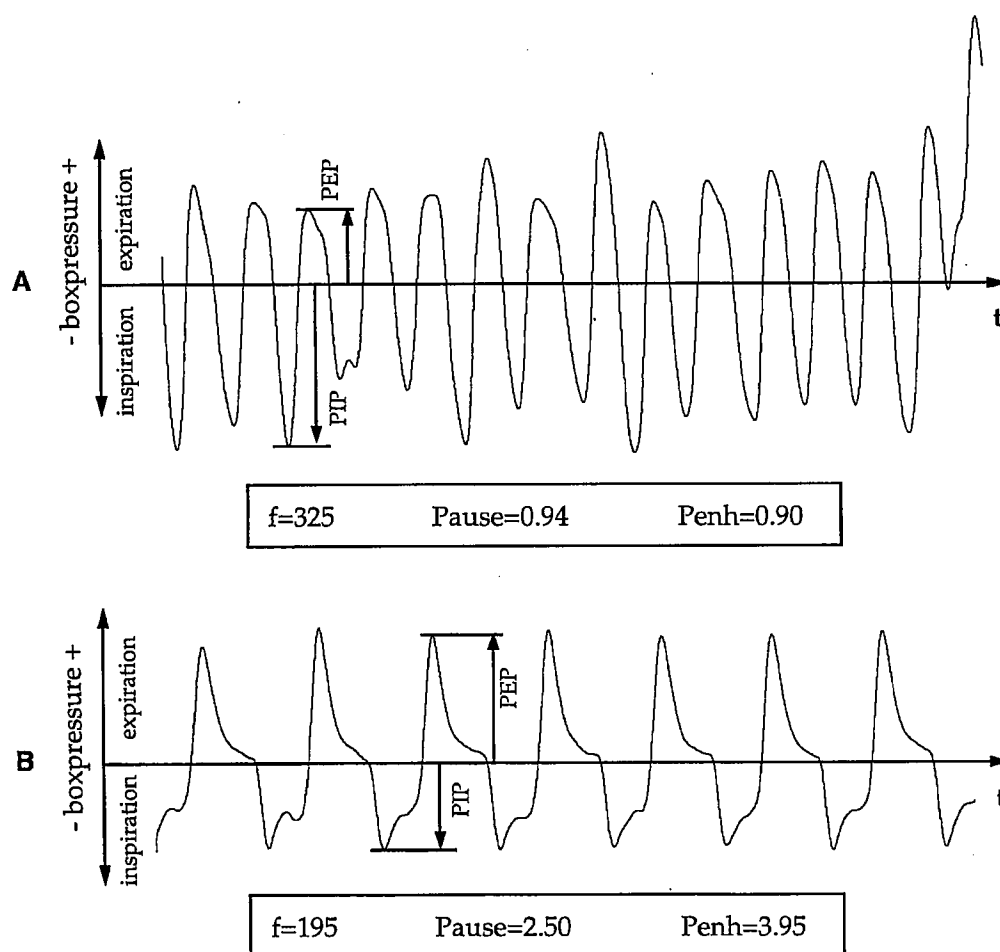


Figure 3. Changes in box pressure waveform after methacholine challenge. Waveform of the box pressure signal derived from a normal mouse after 3 min of nebulization with aerosolized PBS (A) or aerosolized methacholine (50 mg/ml in PBS) (B). f = respiratory rate (breaths/min); Pause, Penh (enhanced pause), PIP and PEP: see Figure 2 for description.

we performed the following studies. Mechanical ventilation was achieved by using a computer-controlled high-speed volume ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). The piston of this ventilator is connected to a linear pumping motor and a linear displacement transducer (21). By measuring the precise position of the shaft and accounting for gas compression, the volume delivered within the mouse or plethysmograph is defined and known. We then ventilated either the empty WBP or the live mice over a frequency (f) rate of 100 to 300 breaths/min and tidal volume ranges of 0.1 to 0.3 ml. We performed a tracheostomy and then connected the mice through the well of the plethysmograph to the ventilator. To investigate the effects of agonist inhalation in ventilated mice, the animals were challenged with MCh (25 mg/ml) given either intratracheally with f set to 60 breaths/min, tidal volume of 500 μ l for 30 s, or intraperitoneally after establishing PBS baselines, and Penh was measured under fixed ventilation settings (f = 300 breaths/min; tidal volume = 150 μ l).

Correlation to Respiratory Rate in Conscious Mice

To investigate if there is a relationship between Penh and respiratory rate in conscious, unrestrained, and spontaneously breathing mice, we investigated the effects of CO₂ inhalation. Normal mice were set in the main chamber of the WBP, and Penh baseline readings were taken for the first 3 min. A steady bias flow with normal air (1 L/min) was established through an additional inlet of the main chamber to allow long-term online readings. A second baseline reading of 3 min was measured after the mice were resting within the box for 30 min. Bias flow was then changed to 8% CO₂ in air (1 L/min), and respiratory rate and Penh were measured after 15 min.

Lower Airway Responsiveness

To document involvement of the lower airways in the measurements of Penh, mice were anesthetized intraperitoneally (0.3 ml 2.5% avertin in PBS) and 5-mm-long sterile plastic tubes were inserted into the tracheas and fixed by suture. PBS, MCh challenge, and measurements were performed in the spontaneously breathing mice in the WBP as described above.

Correlation to Pleural Pressure

In an attempt to directly correlate bronchoconstriction to the index derived by barometric plethysmography, intrapleural pressure changes were measured simultaneously with measurements of Penh. Saline-filled tubes were inserted into the esophagus of anesthetized mice and connected to a pressure transducer (Model MC1; Validyne, Northridge, CA). Mice were challenged with nebulized PBS and increasing concentrations of MCh as above. Changes in intraesophageal pressure (ΔP), which reflect changes in intrapleural pressure, were calculated for each MCh concentration. Changes in box pressure and respiratory frequency were measured by WBP. ΔP and Penh were recorded simultaneously and expressed as a fold increase over values after PBS challenge.

Correlation to Pulmonary Resistance

To correlate Penh with a measurement of lung resistance, *in vivo* pulmonary resistance (R_L) was measured in anesthetized, tracheostomized, and ventilated mice as previously described (14, 15). A four-way connector was attached to the tracheostomy tube, with two ports connected to the inspiratory and expiratory sides of the ventilator (Model 683; Harvard Apparatus, South Natick, MA). Ventilation was achieved with a rate of 160 breaths/min, tidal volume of 150 μ l during recording, and with a rate of 60 breaths/min, tidal volume of 500 μ l during MCh challenge. As a modification to previous work from our laboratory (15), MCh was administered as an aerosol for the period of 10 breaths for each concentration via the tracheostoma. Change in pressure, flow, and volume were recorded, and R_L was calculated from peak values after each challenge. Penh was first measured on Day 31 of the protocol and *in vivo* R_L was obtained in the same animals 1 d later.

Effects of a Beta Agonist

To study the effects of an inhaled β_2 -agonist on measurement of Penh in allergen-sensitized and challenged mice, albuterol was nebulized as an aerosol for 3 min into the main chamber followed by a 3-min read-

ing. The control group consisted of sham-treated mice aerosolized with PBS for 3 min instead of albuterol. Six minutes later, MCh was aerosolized at a 50 mg/ml concentration followed by a 6-min reading. In a different set of experiments, the effects of albuterol on repeated MCh challenge was investigated. After establishing PBS baseline values, mice were challenged with MCh (50 mg/ml) for 3 min and Penh was recorded for 6 min. The mice were divided into two groups receiving nebulization with either PBS or albuterol for 3 min followed 6 min later by a second MCh challenge (50 mg/ml) for 3 min.

Measurement of Anti-OVA Antibody and Total Ig Levels

Anti-OVA Ig serum levels were measured by ELISA as previously described (22). The antibody titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter (EU/ml). Total IgE and IgG levels were determined using the same method compared with known mouse IgE or IgG standards (PharMingen, San Diego, CA). The limits of detection were 100 pg/ml for IgE and 1 ng/ml for IgG.

Bronchoalveolar Lavage (BAL) and Lung Cell Isolation

Lungs were lavaged via a tracheal tube with Hank's balanced salt solution (HBSS, 3 \times 0.5 ml), and the cells in the lavage fluid were counted. Lung cells were isolated as previously described (22). Cells from BAL or lungs were resuspended in HBSS and counted with a hemocytometer. Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting at least 300 cells by light microscopy.

Statistical Analysis

Analysis of variance was used to determine the level of difference between all groups. Single pairs of groups were compared by Student's *t* test. Comparisons for all pairs were performed by Tukey-Kramer HSD test; *p* values for significance were set to 0.05. Values for all measurements are expressed as the mean \pm standard deviation (SD) except for values for airway reactivity (Penh, resistance, impedance), which are presented as the mean \pm standard error of the mean (SEM).

RESULTS

Noninvasive AR Increases after Methacholine Challenge in Allergen-sensitized and Challenged Mice

We established a mouse model of systemic sensitization and airway challenge with allergen, monitoring airway responsiveness using WBP in unrestrained and conscious mice. Sensitization with OVA followed by airway challenge significantly increased serum levels of anti-OVA IgE and IgG₁ and enhanced production of total IgE in BALB/c mice compared with non-sensitized control mice receiving no treatment or OVA airway challenge on two consecutive days (Table 1). In addition, all of the sensitized and challenged mice developed allergen-specific immediate cutaneous responsiveness to intradermal injections of OVA; no responses were observed in nonsensitized control mice without or with airway challenge (data not shown).

We compared the responses to inhaled MCh in the four groups of mice: untreated mice (N), sensitized and PBS-challenged mice (ip), nonsensitized and OVA-challenged mice (Neb), and sensitized and OVA-challenged mice (ipNeb). The control groups (N, Neb, ip) showed similar albeit shallow, dose-dependent increases in Penh in response to aerosolized MCh compared with the Penh values after PBS (Figure 4). In contrast, in mice that were sensitized and challenged with allergen via the airways (ipNeb), the increase in Penh in response to aerosolized MCh was significantly enhanced compared with the control mice. The MCh doses required for 100 and 200% increases in Penh were significantly reduced for sensitized and challenged mice by \sim 3.5-fold and \sim 5-fold, respectively, shifting the dose-responses leftwards compared

TABLE 1
OVA-SPECIFIC ANTIBODY AND TOTAL Ig LEVELS IN THE SERUM

Group	Sensitization	Challenge	OVA-specific (EU/ml) [†]		Total Ig (ng/ml)	
			IgE	IgG ₁	IgE	IgG
N	None	None	< 10	< 10	14.7 ± 1	243 ± 65
Neb	None	OVA	< 10	< 10	16 ± 3	235 ± 68
ipNeb	OVA	OVA	7,622 ± 736 [§]	1,029 ± 369 [§]	48.8 ± 10 [§]	265 ± 86

Definition of abbreviations: OVA = ovalbumin; N = serum titers for OVA-specific and total antibodies, which were determined by ELISA in untreated mice (n = 8); Neb = nonsensitized, OVA-challenged mice (n = 12); ipNeb = OVA-sensitized, OVA-challenged mice (n = 18). Presented are the means ± SD (OVA-specific in ELISA units/ml, total Ig in ng/ml) from three independent experiments.

[†] Presented are the means ± SD from the three independent experiments.

[‡] ELISA units per milliliter.

[§] Significant (p < 0.01) differences compared with control group (N).

with nonsensitized control mice (Table 2). The responses peaked at 1.5 to 2 min after the challenge with aerosolized MCh, and Penh returned to prenebulization values after ~ 3 min for MCh doses ≤ 12 mg/ml and after ~ 5 to 7 min after higher doses. The Penh baseline readings after PBS were similar for all three control groups, but they were higher for sensitized and challenged animals (Figure 4). These data indicate that Penh values are increased in allergen-sensitized, airway-challenged animals. Furthermore, the response to MCh was greater in this group of mice.

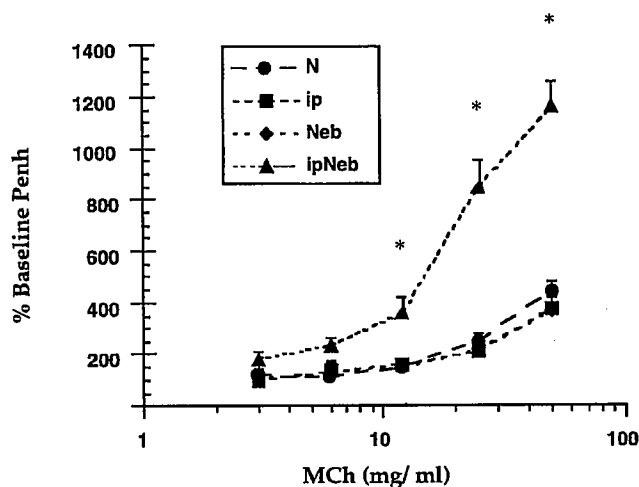


Figure 4. Penh increases in allergen-sensitized and challenged mice. Animals were sensitized with OVA/alum ip on Days 1 and 14 and challenged with OVA via the airways on Days 28, 29, and 30. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber of the WBP and were nebulized first with PBS, then with increasing doses (3 to 50 mg/ml) of methacholine for 3 min for each nebulization, followed by readings of breathing parameters for 3 min after each nebulization with PENH values determined. Compared are nontreated (N) (n = 8), nonsensitized, challenged (Neb) (n = 12), sensitized, nonchallenged (ip) (n = 12), and sensitized, challenged (ipNeb) (n = 12) mice. Expressed are the means ± SEM of the Penh values in percentages of Penh values after PBS nebulization of three independent experiments. Penh_{PBS}: N, 0.84 ± 0.03; Neb, 0.85 ± 0.03; ip, 0.81 ± 0.04; ipNeb, 1.04 ± 0.05; p < 0.05. *p < 0.01 compared with controls values.

Noninvasive AR Increases after Methacholine Challenge in Ventilated Mice

To study the impact of changes in breathing frequencies (f) and tidal volumes, we measured Penh under conditions where mice were mechanically ventilated. First, the empty WBP was connected to the ventilator and Penh was measured over the frequency range of 100 to 300 breaths/min and the tidal volume range of 100 to 300 µl. Both f and tidal volume were measured correctly by the WBP, and recorded values for tidal volume did not change under different f. Penh during mechanical ventilation changed less than 10% with various f in measurements performed in live, ventilated mice. Penh values increased nearly proportionally with increasing tidal volume in a range from 100 to 250 µl. The < 2-fold increase in Penh observed in ventilated mice (0.26 at 100 µl to 0.44 at 250 µl) was due to an increase in the Pause (0.34 to 0.54) resulting from a decrease in Tr (from 0.09 s at 100 µl to 0.07 s at 250 µl) and to a lesser extent by a decrease of T_E (from 0.12 s to 0.11 s). The decrease in Tr may be explained by the greater elastic recoil/smaller compliance of the lungs when ventilated with a higher tidal volume. These data further indicate that the volume dependency of Penh does not account for the much greater changes (> 10-fold increase versus baseline Penh) seen in sensitized challenged mice with similar changes in tidal volume (see Table 3).

Next, we measured the changes in Penh after challenge with MCh under ventilated conditions. Normal, anesthetized mice were ventilated and challenged with MCh (25 mg/ml) either intratracheally or intraperitoneally. Under both conditions, the mice developed a more than 200% increase in Penh

TABLE 2
DOSE-RESPONSE OF PENH TO MCH

Group	Sensitization	Challenge	MCh Dose (mg/ml) Required for an Increase in Penh of			
			100%	(LL; UL)	200%	(LL; UL)
N	None	None	17.7	10.5; 16	32	21; 48.5
Neb	None	OVA	18.7	11.5; 30.4	42	32; 105
ipNeb	OVA	OVA	5.2 [†]	4.4; 6.1	7 [†]	5.6; 8.3

Definition of abbreviations: Penh = plethysmography and increases in enhanced pause; MCh = methacholine; N = dose-response in Penh after challenge with aerosolized MCh in untreated mice (n = 8). For other definitions, see Table 1.

[†] Reported is the geometric mean and the lower (LL) and upper (UL) level of the 95% confidence interval in mg/ml of MCh required for an increase of Penh to 100 or 200% compared with PBS-baseline values.

[‡] p < 0.01 versus control groups (N and Neb).

TABLE 3
EFFECTS OF METHACHOLINE OR AVERTIN ON
BREATHING PATTERNS AND PENH*

Group	Anesthesia	Challenge (nebulized)	Respiratory Frequency	Tidal Volume	Penh
ipN	None	PBS	320 ± 12 [†]	0.25 ± 0.03 [†]	1.01 ± 0.06 [‡]
ipN	None	MCh 50	165 ± 24	0.4 ± 0.04	11.4 ± 2.6
ipN	Avertin	PBS	189 ± 21	0.35 ± 0.04	1.04 ± 0.2

* Correlation between breathing patterns and Penh values. Expressed are the mean ± SD values of respiratory frequency (in breath/min), tidal volume (in μ l), and Penh after PBS and MCh (50 mg/ml) challenge for conscious (n = 8) and anesthetized (avertin, n = 8), sensitized, and challenged mice.

[†] p < 0.05 versus two other groups.

[‡] p < 0.05 versus MCh challenge.

values after MCh of PBS-baseline values. This suggests that the increase in Penh cannot be accounted for by changes in f or tidal volume after challenge with MCh as these variables are controlled in mechanically ventilated mice.

Noninvasive AR Does not Correlate with Breathing Patterns and Respiratory Rate

MCh challenge of mice induces increases in Penh and decreases in respiratory rate. To study the effects of changes in breathing patterns on Penh in spontaneously breathing mice and to investigate if the observed slowing of the respiratory rate itself causes an increase in Penh, sensitized and challenged mice were anesthetized intraperitoneally with an injection of avertin (2.5% in PBS) and compared with conscious animals. In anesthetized animals, frequency was decreased to ~60% compared with that in conscious mice. However, changes in these breathing patterns were not accompanied by increases in Penh

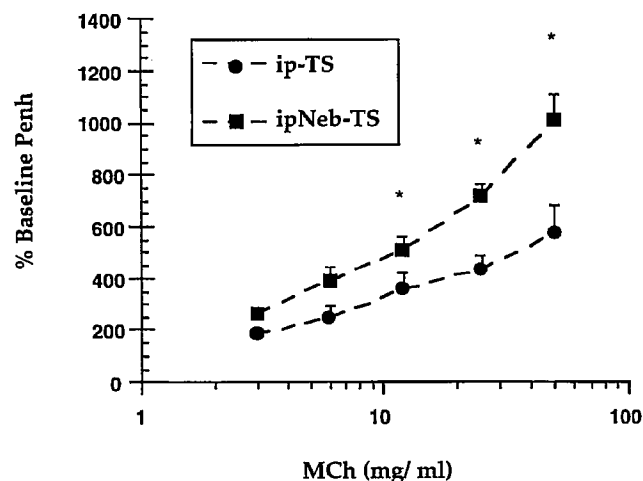


Figure 5. Penh increases after methacholine challenge of the lower airways. Mice were sensitized and challenged as described in Figure 1. Mice were tracheostomized, and airway responsiveness was measured as described in Figure 1. Compared are sensitized but not challenged (ip-TS) (n = 12) and sensitized and challenged tracheostomized mice (ipNeb-TS) (n = 12). Expressed are the means ± SEM of the Penh values in percentages of Penh values after PBS nebulization from three independent experiments. Penh_{PBS}: ip-TS, 0.94 ± 0.07; ipN-TS, 1.2 ± 0.15. *p < 0.01 compared with controls values.

(Table 3). MCh challenge (50 mg/ml) of conscious mice resulted in changes in breathing patterns similar to those observed in anesthetized animals, but they were followed by significant increases in Penh. Challenge of anesthetized mice with MCh resulted in a dose-response curve similar to those seen in the conscious animals (Figures 4 and 5), although the respiratory rate of each concentration of MCh challenge in the anesthetized animals was less than that in conscious animals (data not shown).

To further study the influence of the respiratory rate in spontaneously breathing mice receiving Penh, CO₂ was used as a respiratory stimulant. After 30 min resting in the box with a steady bias flow of normal air, mice showed a significant decrease in the average respiratory rate of 25% with virtually no changes in Penh readings (Table 4). Changing the bias flow from normal air to air containing 8% CO₂ induced a significant increase in respiratory rate of 46% accompanied with a nonsignificant drop of Penh by 10% (p = 0.2). These data indicate that decreases or increases in the respiratory rate are not necessarily accompanied with changes in Penh but rather are independently regulated.

Noninvasive AR Increases after Methacholine Challenge of the Lower Airways

To address the possibility that increases in Penh are simply due to reactions of the upper airways, e.g., swelling of the nasal mucosa or increased glandular activity, we bypassed the upper airways by performing a tracheostomy (TS) in mice before challenge with aerosolized MCh. As indicated in Figure 5, challenge of these mice with nebulized MCh resulted in dose-dependent increases in Penh. In sensitized and challenged mice (ipNeb-TS), increases in Penh values were significantly enhanced. Baseline Penh values were similarly higher in ipNeb-TS than in ip-TS animals (Figure 5). The higher Penh baseline values compared with those in nontracheostomized mice is most likely due to the fixed resistance of the tracheostomy. The magnitude of the dose-response in sensitized, challenged animals after challenge with higher doses of MCh (25 and 50 mg/ml) was very similar in tracheostomized and nontracheostomized mice (Figures 4 and 5). The MCh dose required for a 100% increase in Penh was significantly decreased and shifted to the left from 4.4 (3.3; 5.9) in ip-TS to 2.9 (2.3; 3.7) in ipNeb-TS (~1.5-fold, p < 0.05), and from 16 (8; 24) in ip-TS to 4.6 (2.9; 7.8) in ipNeb-TS (~3.5 fold, p < 0.01) for a 200% increase. The smaller magnitude of the shift in that dose-response to MCh in tracheostomized compared with nontracheostomized mice might be explained by the fact that the availability of MCh is higher in tracheostomized mice because the upper airways are by-passed, which may result in higher responses in nonsensitized mice. Secondly, the data suggest that at least

TABLE 4
EFFECT OF CO₂ ON RESPIRATORY RATE AND PENH*

Condition	Time in Box (min)	Air (bias flow)	Breathing Frequency	Penh
1	3	Air	306 ± 13	1.06 ± 0.17
2	30	Air	243 ± 15 [†]	1.07 ± 0.15
3	45	Air/8% CO ₂	356 ± 13 [‡]	0.95 ± 0.2

* Correlation between respiratory frequency and Penh values. Expressed are the mean ± SD values of respiratory frequency (in breaths per min) and Penh for mice (n = 4) 3 min (Condition 1) and 30 min (Condition 2) after start of online measurements and after additional 10 min of bias flow with 8% CO₂ in air (Condition 3).

[†] p < 0.01 versus condition 1.

[‡] p < 0.01 versus Condition 2.

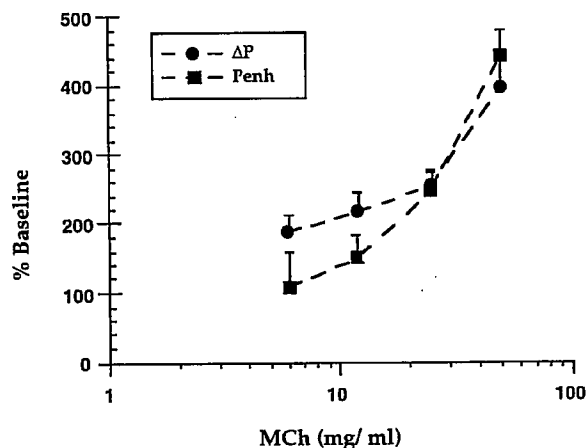


Figure 6. Increases in Penh and intrapleural pressure after methacholine airway challenge. Measurements of airway responsiveness in the WBP were performed as described in Figure 1. A saline-filled esophageal tube was connected to a second pressure transducer, and intraesophageal pressure was recorded simultaneously with PENH after each challenge with MCh. Expressed are the means \pm SEM of Penh and ΔP (intraesophageal pressure differences) as percentages of baseline values after PBS nebulization from two independent experiments ($n = 8$).

part of the increased responsiveness measured by Penh may be related to altered responsiveness of the upper airways.

Noninvasive AR Correlates with Intrapleural Pressure

To directly correlate Penh values with changes in pleural pressure occurring in the lower airways after MCh challenge, a saline-filled esophageal tube was placed in the mice to reflect changes in intrapleural pressure. Simultaneously, Penh was measured using the WBP. Mice were then challenged with aerosolized PBS or increasing doses of MCh. As shown in Figure 6, nebulized MCh induced increases in Penh similar to those in intrapleural pressure (ΔP) compared with values after PBS exposure. Moreover, the increases in Penh correlated with increases in ΔP (Figure 7B). These data demonstrate that Penh values correlate with increases in intrapleural pressure differences in the lower airways after MCh challenge.

Noninvasive AR Correlated with *in vivo* Pulmonary Resistance

To determine if increases in Penh values correlate with increased *in vivo* pulmonary resistance, we monitored Penh and pulmonary resistance in the same animals on 2 consecutive days. Pulmonary resistance was measured in intubated and ventilated mice, administering aerosolized MCh via the tracheostomy. Data were calculated from the peak values after each MCh challenge and expressed as the increase compared with measurements after PBS nebulization. Aerosolized MCh increased pulmonary resistance in a dose-dependent manner; sensitized, OVA-challenged animals (ipNeb) showed significantly higher pulmonary resistance than did nonsensitized, OVA-challenged control animals (Neb) (Figure 8B). Increases in pulmonary resistance paralleled increases in Penh values monitored by WBP in the same animals the day previously (Figure 8A). A comparison of the responses of R_L and Penh for individual mice in the same experiment (Figure 7A) indicates the

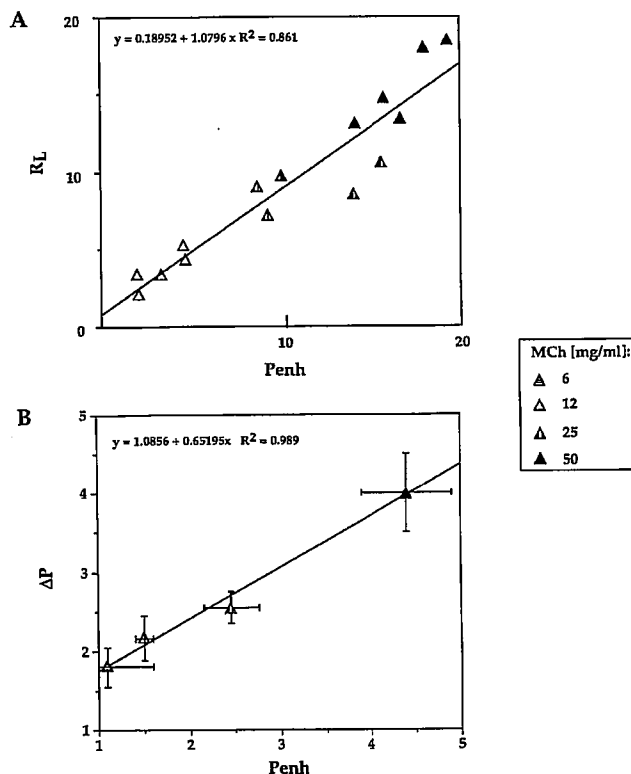


Figure 7. Penh correlates with pulmonary resistance and intrapleural pressure. Mice were sensitized and measurements of airway responsiveness were performed as described in Figure 4. Compared are the responses to aerosolized MCh measuring Penh and airway resistance (R_L) on 2 consecutive days in the same individual mice (A), or measuring Penh and ΔP simultaneously as described in Figure 3 (B). Shown are the results from one of two similar experiments (A) and the mean \pm SEM of the results from two independent experiments (B), respectively.

strong correlation between Penh and increased pulmonary resistance in sensitized and challenged mice.

Noninvasive AR Increases Are Inhibited by Albuterol

To assess the effect of β_2 -agonist on Penh, albuterol was administered by nebulization to allergen-sensitized and challenged mice after obtaining a PBS baseline. Aerosolization of albuterol did not change baseline Penh values after PBS (data not shown). MCh was then aerosolized at 50 mg/ml, and Penh was recorded for 6 min after each nebulization. Sensitized and allergen-challenged animals receiving sham-treatment with aerosolized PBS instead of the β_2 -agonist showed a significant increase in Penh values after MCh challenge (Figure 9). Pretreatment with aerosolized albuterol significantly reduced the increases in Penh values after MCh challenge. Subsequent albuterol treatment of mice that showed a more than 10-fold increase in Penh after a first MCh (50 mg/ml) challenge resulted in significantly reduced Penh values after a second MCh challenge ($650 \pm 120\%$ of PBS baseline), whereas mice receiving PBS sham-treatment instead of albuterol after a first MCh challenge showed consistently higher Penh readings after repeated MCh challenge ($1,250 \pm 150\%$ of PBS baseline). These

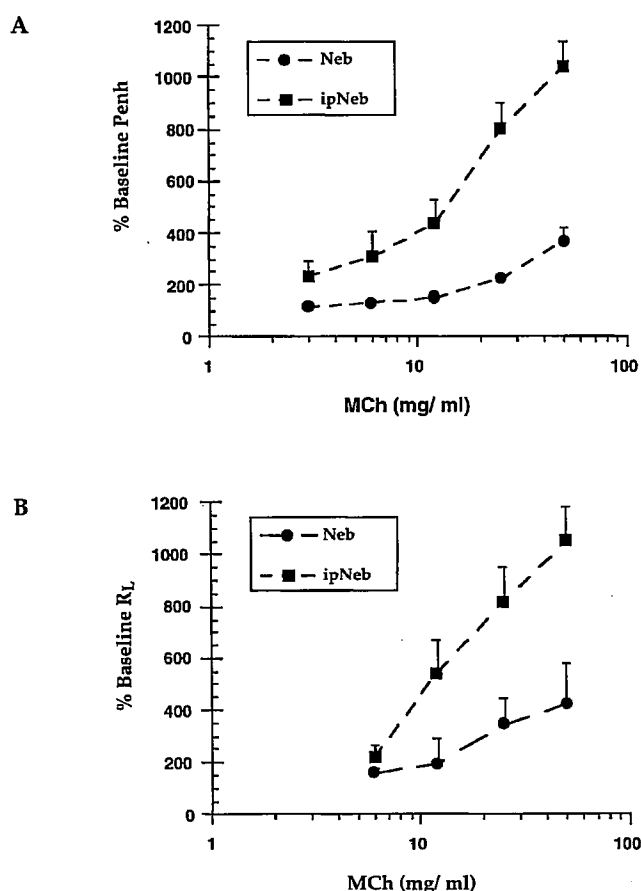


Figure 8. Increases in Penh and pulmonary resistance after methacholine airway challenge. Mice were sensitized and measurements of airway responsiveness were performed as described in Figure 1. Using the same mice, pulmonary resistance was measured in anesthetized, tracheostomized, and ventilated animals the following day. Aerosolized PBS and MCh were administered via the tracheostomy. Pulmonary resistance was calculated as $R_L = \Delta P$ (difference in tracheal pressure)/ ΔV (flow change) from peak values after each challenge. Compared are nonsensitized, challenged (Neb) ($n = 12$) and sensitized, challenged (ipNeb) ($n = 12$) mice. Expressed are the means \pm SEM of Penh (A) and of R_L (B) as percentages of baseline values after PBS nebulization. $Penh_{PBS}$: Neb, 0.86 ± 0.03 ; ipNeb, 1.09 ± 0.08 from three independent experiments * $p < 0.01$ compared with control (N).

data indicate that increases in Penh values in response to MCh are at least partially preventable after pretreatment with the bronchodilator.

Increases in Penh Are Associated with Increased Eosinophils in Lung Tissue

To correlate AR with airway inflammation, total leukocyte counts and differential counts for BAL fluid cells and isolated lung cells of individual mice were compared in the different groups. Sensitization and challenge resulted in a significant increase in eosinophils in BAL fluid ($38 \pm 4\%$) and in lung cells ($13 \pm 2\%$) compared with naive animals ($1.2 \pm 0.4\%$ in BAL and $1.1 \pm 0.5\%$ in lung cells). The increase in total numbers of eosinophils of 12-fold in lung cells and 70-fold in BAL fluid

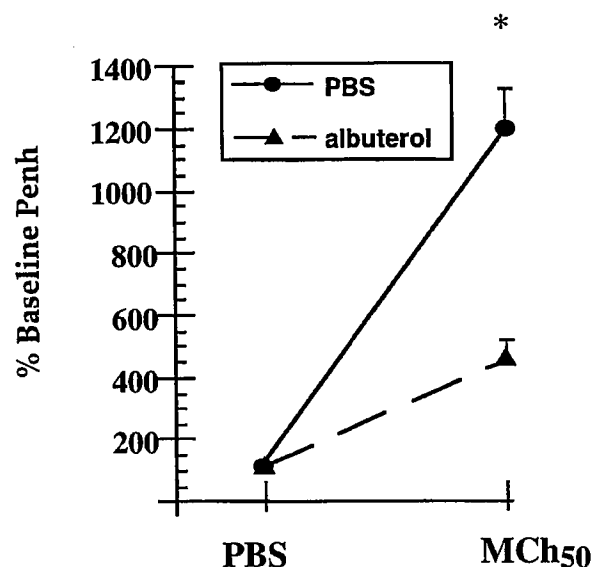


Figure 9. Penh increases are inhibited by nebulized albuterol. Mice were sensitized, and measurements of airway responsiveness were performed as described in Figure 1. After obtaining baseline Penh values after aerosolization of PBS, mice were treated with aerosolized albuterol or PBS for 3 min. After 6 min, mice were challenged with aerosolized MCh at 25 and 50 mg/ml for 3 min each, and Penh was recorded for 3 and 6 min after each nebulization. Compared are sensitized, challenged mice with albuterol ($n = 4$) or PBS ($n = 4$) treatment. Expressed are the means \pm SEM of Penh values in percentages of Penh values after PBS nebulization. $Penh_{PBS}$, 1.06 ± 0.03 ; albuterol, 1.09 ± 0.08 . * $p < 0.01$ compared with PBS sham-treated mice.

was associated with a ~ 4 -fold increase in Penh after similar MCh challenge compared with the control animals (Table 5).

DISCUSSION

In this report, we characterize a method to measure *in vivo* airway responsiveness in conscious, spontaneously breathing mice. We used a barometric whole-body plethysmograph (WBP) that measures pressure differences between a main chamber containing the animal and a reference chamber. This box pressure signal potentially detects a number of different parameters (23). Among these are heat and humidity changes that occur in the inspired and expired air. However, as the respiratory rate of the animal is 300 breaths/min (or ~ 5 Hz) and stays at ~ 120 breaths/min (or ~ 2 Hz) in bronchoconstricted animals, the

TABLE 5
INCREASES IN PENH CORRELATE WITH EOSINOPHIL LUNG INFILTRATION*

Group	Sensitization (ip)	Challenge (nebulized)	Eosinophils In		Penh After	
			BAL ($\times 10^3$)	Lungs ($\times 10^6$)	MCh 25	MCh 50
N	None	None	2.4 ± 1.3	0.7 ± 0.2	1.4 ± 0.3	2.8 ± 0.2
Neb	None	OVA	2.2 ± 1.1	0.7 ± 0.3	1.5 ± 0.2	2.9 ± 0.4
ipNeb	OVA	OVA	$173 \pm 65^\dagger$	$8.6 \pm 2.2^\dagger$	$5.3 \pm 0.9^\dagger$	$9.06 \pm 1^\dagger$

* Correlation between eosinophil numbers in the BAL fluid and the lung tissue and Penh values after allergic sensitization. Compared are nontreated (N), nonsensitized, OVA-challenged (Neb), and sensitized, challenged (ipNeb) mice. Presented are the means \pm SEM.

† Significant ($p < 0.05$) differences compared with control groups (N and Neb).

volume/flow changes are, in all likelihood, isothermal. The remaining contributors to the changes indicated by the changes of the box pressure signal are alterations in respiratory rate, tidal volume, or compression artifacts (24) (phase lags between nasal and thoracic flow). The avertin (Table 3) and CO₂ (Table 4) experiments suggest that changes in the respiratory rate are not causing similar changes in Penh as observed after agonist inhalation. Independence of Penh measurements from breathing frequency was determined in studies in mechanically ventilated mice where Penh is largely independent of frequency under ventilation with constant volumes. As some increase in Penh occurs with increasing tidal volume in mechanically ventilated mice, yet these changes cannot account for the more than 10-fold increase in Penh values observed after MCh challenge in allergen-sensitized and challenged mice. Taken together, these data suggest that this technique can be used to assess airway responsiveness in mice in a noninvasive fashion.

Bronchoconstriction is known to alter breathing patterns, and indeed changes in Pause and Penh are really due to alterations in the timing of breathing as well as a prolongation of the expiratory time. Airway constriction is further known to lead to an increase in the thoracic flow that is not synchronized with the nasal flow (25), thus resulting in an increase in the box pressure signal. The increase in the time lag between the nasal and the thoracic flow is proportional to total airway resistance and can be used to measure AR by barometric plethysmography (26–28). Penh is considered an empiric parameter that reflects changes in the waveform of the measured box pressure signal that are a consequence of bronchoconstriction. The data in this report show a close correlation between changes in indices derived from the box pressure signal (Penh) and changes in intrapleural pressure or lung resistance (R_L) to aerosolized MCh. Therefore, we conclude that under these conditions, this measurement (Penh) appears to be a valid indicator of bronchoconstriction in mice.

Several investigators have used barometric WBP to measure AHR in guinea pigs and rats (18, 26, 27). The use of this technique in mice enabled us to establish dose-response curves to an aerosolized bronchoconstrictive agent and to differentiate between normal levels of AR in control animals and hyperresponsiveness in allergen-sensitized and challenged mice. Sensitization without allergen challenge or airway challenge of nonsensitized mice was without effect on the Penh values when compared with nontreated animals. The response to MCh in sensitized and challenged animals was both shifted to the left (Table 2) and amplified (elevation of the maximal response, Figure 4) compared with that in control animals. This resembles *in vivo* AHR in patients suffering from bronchial asthma (29) and measurements of AHR with invasive methods in other animal models (30). The most likely mechanism underlying the increases in Penh is bronchoconstriction, mediated through the muscarinic receptors on smooth muscles of the airways. This is supported by the rapid but transient responses to aerosolized MCh. Further evidence for airway obstruction as the major mechanism underlying the increases in Penh was obtained using a β_2 -agonist: pretreatment of sensitized and allergen challenged animals with aerosolized albuterol significantly reduced the increases in Penh after MCh challenge. Albuterol treatment of mice that had already responded with high increases in Penh values after a first dose of MCh prevented similar responses after a second challenge with the same concentration of MCh. Importantly, changes in the respiratory rate in anesthetized mice or after CO₂-stimulation were not accompanied by changes in the Penh values, suggesting that Penh does not correlate simply with changes in breathing patterns.

One problem of measuring AHR with barometric WBP is the uncertainty of the site of obstruction (31) and the absolute value of airway resistance. We studied the effects of lower airway challenge on the development of AHR in the WBP. Lower airway challenge with MCh in tracheostomized mice resulted in a significant increase in airway responses and a shift to the left of the dose-response in allergen-sensitized and challenged animals compared with control mice. The somewhat smaller magnitude in the shift of the MCh dose-response when the upper airways are by-passed by the tracheostomy might suggest that at least a small part of the increased responsiveness as measured with Penh is related to altered upper airway responsiveness. Direct correlation between Penh and changes in lower AR was achieved in parallel measurements of Penh and intrapleural pressure after aerosolized MCh challenge. In order to correlate Penh values with pulmonary resistance, we compared the responses measured by WBP with measurements of pulmonary resistance in the same animals, obtained 1 d later. The responses monitored in the two systems were virtually identical, with comparable increases and a similar left-shift of the dose-response curve over baseline values. These data indicate that Penh correlates well with measurements of pulmonary resistance, and that WBP provides a valid measurement of AHR in allergen sensitized and challenged mice.

A number of studies have associated changes in AHR with increases in allergen-specific IgE (32, 23) and eosinophil airway infiltration (34–36). In our model of allergic sensitization, increases in specific IgE were observed. Further, after allergen challenge, increased numbers of eosinophils were detected in the BAL fluid and in isolated lung cells. Increases in Penh values were associated with production of antigen-specific IgE and the development of an eosinophil infiltration in the lungs after allergen challenge of sensitized mice. These findings confirm the association between AHR, measured by barometric WBP, and eosinophilic inflammation.

In summary, this report describes a method to monitor AHR to aerosolized MCh challenge in conscious, spontaneously breathing mice after allergen-sensitization and challenge. We have shown that changes in the box pressure signal (or the empirically derived parameter of Penh) track the changes in the respiratory system caused by bronchoconstriction. Because barometric WBP is a noninvasive technique the animals do not need to be killed once the measurements are finished, and several measurements on the same animals can be performed, allowing longitudinal studies and investigation of treatment protocols. AHR can be monitored over an extended period of time to mimic chronic allergen exposure, and the kinetics of restoration and secondary responses to allergen can be studied. In addition, this technique is potentially attractive in studying animals infected with various pathogens. Measurements of increased AHR obtained in the WBP correlated with increases in IgE serum levels, eosinophil lung infiltration, and increased lung resistance. We conclude that WBP provides a promising technique to investigate the mechanism and the kinetics underlying the development of AHR and will support the study of new approaches in the prevention of AHR.

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EXHIBIT C

Development of Eosinophilic Airway Inflammation and Airway Hyperresponsiveness in Mast Cell-deficient Mice

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Summary

Mast cells are the main effector cells of immediate hypersensitivity and anaphylaxis. Their role in the development of allergen-induced airway hyperresponsiveness (AHR) is controversial and based on indirect evidence. To address these issues, mast cell-deficient mice (*W/W^u*) and their congenic littermates were sensitized to ovalbumin (OVA) by intraperitoneal injection and subsequently challenged with OVA via the airways. Comparison of OVA-specific immunoglobulin E (IgE) levels in the serum and numbers of eosinophils in bronchoalveolar lavage fluid or lung digests showed no differences between the two groups of mice. Further, measurements of airway resistance and dynamic compliance at baseline and after inhalation of methacholine were similar. These data indicate that mast cells or IgE-mast cell activation is not required for the development of eosinophilic inflammation and AHR in mice sensitized to allergen via the intraperitoneal route and challenged via the airways.

Mast cells play a central role in immediate allergic reactions (1) and in the early phase of the asthmatic response, but their role in the late phase response or sustained airway hyperresponsiveness is not clearly defined. Arming and activation of mast cells is through the binding of IgE to high affinity IgE receptors (FcεRI) on the cell surface (2, 3). After antigen cross-linking, the cells discharge a group of mediators including histamine and leukotrienes (4, 5), which trigger immediate responses. The fact that mast cells may also synthesize and secrete several cytokines on activation, including IL-4 and TNF-α, indicates their potential role in the sustained airway abnormalities. It is largely based on circumstantial evidence that mast cells are implicated in asthma pathogenesis. Because many other cell types express high or low affinity receptors for IgE and can release biologically active mediators on activation (6), a number of other cell types may be important in the IgE-dependent responses in the airways.

Mast cell-deficient mice can be used to directly assess the role of mast cells in allergen-driven airway hyperresponsiveness (AHR) (7). However, there have been limited investigations of these mice in terms of airway inflammation and the development of AHR. In the present study, we assessed the physiological response of the airways after sensitization and challenge to OVA in *W/W^u*, genetically mast cell-deficient mice to investigate more directly the role of the mast cell.

Materials and Methods

Animals. Female mast cell-deficient ([WB/Rej-kit^W/+ × C57BL6J-kit^W-v/+]F1 - [W/W^u]mice) (*W/W^u*) and congenic WBB6F1 normal mice (+/+) from 8 to 12 wk of age were obtained from Jackson Labs. (Bar Harbor, ME). The animals were maintained on an OVA-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Sensitization and Airway Challenge. Groups of mice (6–10 mice/group/experiment) receiving the following treatment were studied: (a) airway challenge to nebulized OVA alone (N); (b) sensitization to OVA with alum plus aerosolized airway challenge with nebulized OVA (ipN). Mice were immunized by intraperitoneal injection of 20 μg of OVA (Grade V; Sigma Chemical Co., St. Louis, MO) emulsified in 2.25 mg alum (AlumImject; Pierce, Rockford, IL) in a total volume of 100 μl on days 1 and 14. Mice were challenged via the airways with OVA (1% in saline) for 20 min on days 28, 29, and 30 by ultrasonic nebulization, and assessed 48 h after the last OVA exposure for AHR.

Determination of Airway Responsiveness. Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine (MCh) via the airways. Anesthetized, tracheostomized mice were mechanically ventilated and lung function was assessed using methods similar to those described by Martin et al. (8). A four-way connector was attached to the tracheostomy tube, with two ports connected to the inspiratory and expiratory sides of a ventilator (model 683; Harvard Apparatus, South Natick, MA). Ventilation was achieved at 160 breaths/min

and a tidal volume of 0.15 ml with a positive end-expiratory pressure of 2–4 cm H₂O.

The Plexiglas chamber containing the mouse was continuous with a 1.0-liter glass bottle filled with copper gauze to stabilize the volume signal for thermal drift. Transpulmonary pressure was detected by a pressure transducer with one side connected to the fourth port of the four-way connector and the other side connected to a second port on the plethysmograph. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer and then referenced to a second copper gauze-filled 1.0-liter glass bottle. Flow was measured by digital differentiation of the volume signal. Lung resistance (R_L) and dynamic compliance (C_{dyn}) were continuously computed (Labview, National Instruments, TX) by fitting flow, volume, and pressure to an equation of motion.

Aerosolized agents were administered for 10 s with a tidal volume of 0.5 ml (9). From 20 s up to 3 min after each aerosol challenge, the data of R_L and C_{dyn} were continuously collected. Maximum values of R_L and minimum values of C_{dyn} were taken to express changes in murine airway function.

Bronchoalveolar Lavage and Lung Cell Isolation. Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS (1 × 1 ml 37°C). The volume of collected bronchoalveolar lavage (BAL) fluid was measured in each sample and numbers of leukocytes were counted (Coulter Counter; Coulter Corporation, Hialeah, FL). Cells in lung tissue were isolated and counted as previously described (10).

Histologic and Immunohistochemistry Studies. After perfusion via the right ventricle, lungs were inflated through the trachea with 2 ml air and then fixed in 10% formalin by immersion. Blocks of the left lung tissue were cut from around the main bronchus and embedded in paraffin blocks, 5-μm tissue sections were affixed to microscope slides and deparaffinized. The slides were then stained with Astra Blue/Vital New Red and mast cells and eosinophils were examined under light microscopy (11).

Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using a rabbit anti-mouse MBP (provided by Dr. G. Gleich and Dr. J. Lee, Mayo Clinic, Rochester, MN and Scottsdale, AZ, respectively) (12). Numbers of eosinophils in the submucosal tissue around central airways were analyzed using the

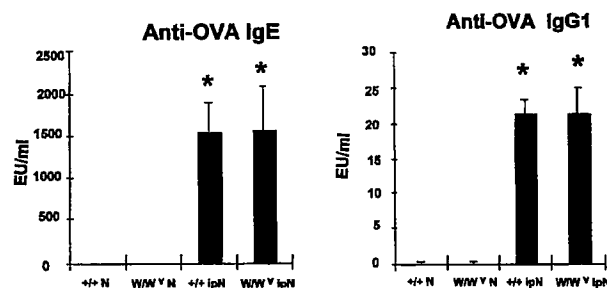


Figure 1. OVA-specific antibody in the serum. Serum titers for OVA-specific antibodies in +/+ and W/W^y mice were determined after sensitization and challenge ($n = 7$) compared with mice receiving challenge alone ($n = 6$). The results for each of the groups are expressed as means \pm SEM. *Significant differences ($P < 0.05$) between the groups (N versus IpN). EU, ELISA units; N, challenge (nebulization alone); IpN, sensitization and challenge.

IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh counting four different sections per animal (12).

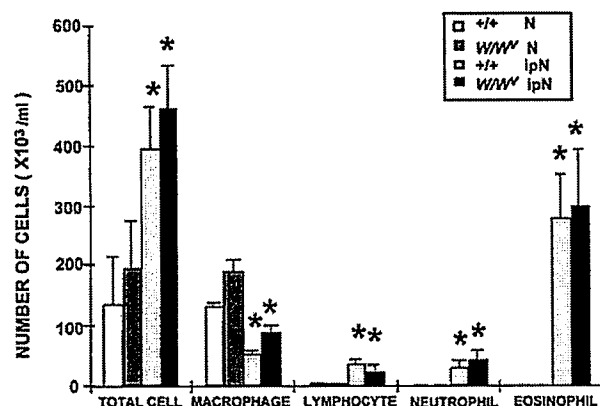
Measurement of Anti-OVA Antibody. Serum levels of anti-OVA IgG1 and IgE were measured by ELISA as previously described (10).

Statistical Analysis. All results are expressed as the mean and SEM. Analysis of variance was used to determine the levels of difference between all groups. Pairs of groups were compared by unpaired two-tailed Student's t test. ANOVA was used to compare percent changes of R_L and C_{dyn} between different strains with the same treatment. The p values for significance were set to 0.05.

Results

Antibody Responses to OVA Sensitization and Challenge. As shown in Fig. 1, serum levels of anti-OVA IgE and IgG1 were comparable in the mast cell-deficient mice and

a BAL



b LUNG DIGESTION

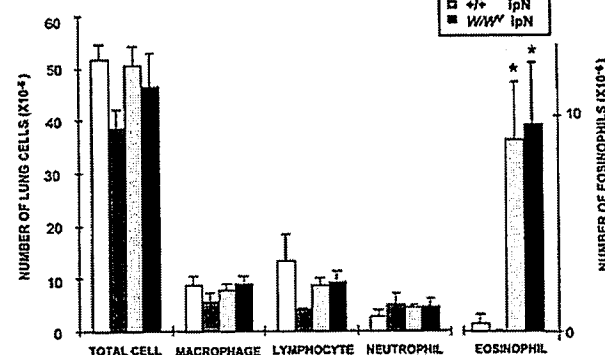


Figure 2. (a) Cellular composition of BAL fluid. Mice were sensitized and challenged as described in Materials and Methods. BAL fluid was obtained from the same groups described in the legend to Fig. 1. The results for each group are expressed as means \pm SEM. *Significant differences ($P < 0.05$) between the groups (N versus IpN). (b) Cellular composition of isolated lung cells. Lung cells were prepared from animals sensitized and challenged as described in the legend to Fig. 1. The results for each group are expressed as means \pm SEM ($n = 4$ /group). *Significant differences ($P < 0.05$) between the groups (N versus IpN).

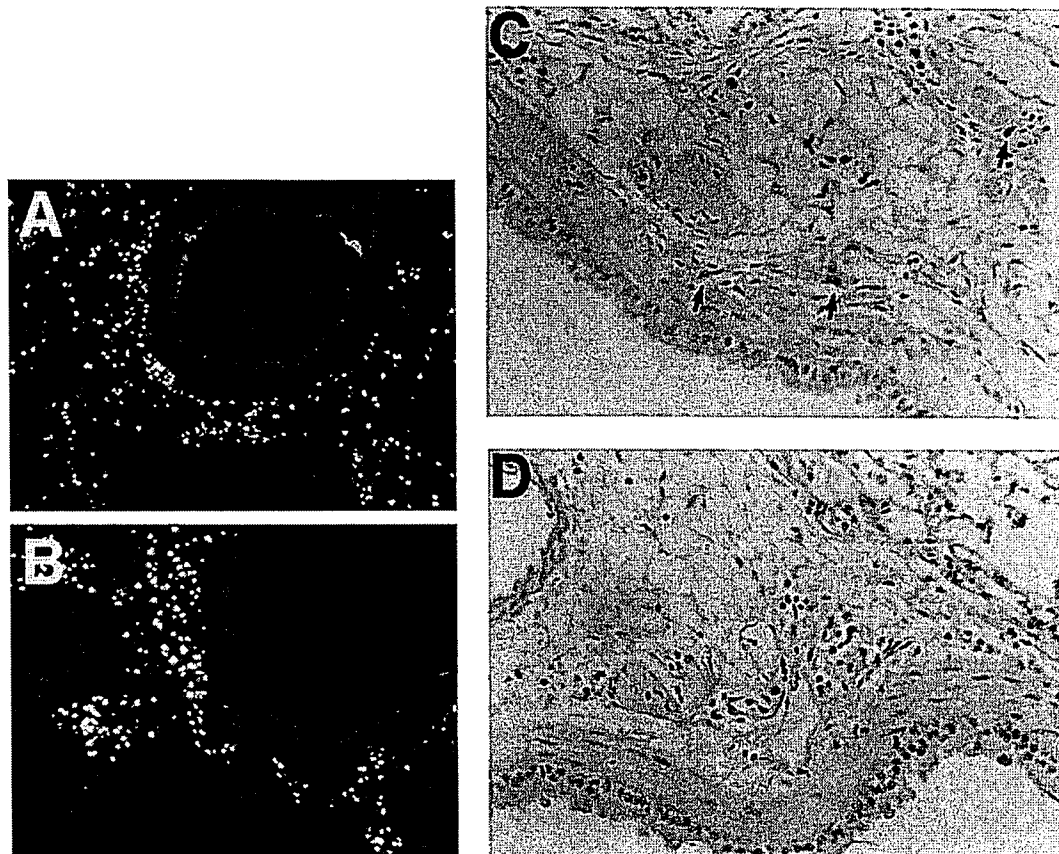


Figure 3. Immunohistochemistry of peribronchial tissue after sensitization and challenge with OVA. Localization of eosinophils and mast cells are shown. $+/+$ mice are shown in *a* and *c* and W/W^v mice in *b* and *d*. In *c* and *d*, cells were stained with Astra Blue/Vital New Red. For *a* and *b*, staining was with a rabbit anti-mouse MBP antibody and fluorescein-labeled goat anti-rabbit IgG. Original magnification of 500.

the congenic littermates after sensitization and challenge with OVA. Challenge alone on three occasions was insufficient to trigger antibody responses in either group of mice.

Eosinophilic Accumulation in the BAL and Lung. As shown in Fig. 2 *a*, sensitization and challenge with OVA had a marked effect on the numbers and composition of the cells recovered. In both groups of mice, macrophages were the predominant cell type in the mice receiving three challenges with OVA alone (similar to control mice; data not shown). However, after both sensitization and challenge, cell numbers increased and the predominant cells in the BAL were eosinophils, comprising roughly 60% of the cells in both the mast cell-deficient and congenic littermates.

When lung digests were examined, sensitization and challenge also resulted in a marked increase in eosinophil numbers (Fig. 2 *b*), although total cell numbers were little changed when compared with challenge alone. As in the BAL, there were no differences in the numbers of eosinophils in the lung digests between mice that were mast cell deficient or sufficient.

Localization of Eosinophils and Mast Cells in Lung Tissue. After staining with anti-MBP antibody, sensitization and

challenge significantly increased the numbers of eosinophils per area in the peribronchial tissue of both groups of mice (Fig. 3, *a* and *b*) to $187 \pm 23/\text{mm}^2$ in $+/+$ mice, $168 \pm 18/\text{mm}^2$ in W/W^v mice ($n = 4$). In animals challenged alone very few eosinophils were detected in these sites ($13 \pm 4/\text{mm}^2$). Staining with Astra Blue/Vital New Red revealed the accumulation of mast cells in the submucosal tissue of the bronchi in sensitized and challenged $+/+$ mice (Fig. 3 *c*). None could be identified in any of the sections examined from W/W^v mice (Fig. 3 *d*).

Airway Responsiveness. We examined baseline lung function and assessed the airway response to inhaled methacholine. Baseline (before MCh challenge) measures of lung function as assessed with RL and Cdyn are presented in Table 1. The values in all four groups were comparable. The response to aerosolized methacholine in mice that were challenged with antigen alone revealed small, dose-dependent changes in RL and a 20–30% dose-dependent fall in Cdyn (Fig. 4). After sensitization and challenge, resistance values increased by almost fivefold and dynamic compliance was reduced by 60–70% with comparable doses of methacholine. The responses in the mast cell-deficient mice, if any-

Table 1. Baseline Values of RL and Cdyn in Mice

Mice	Group	RL	Cdyn
		<i>cmH₂O</i> · <i>ml</i> ⁻¹ · <i>sec</i>	<i>ml</i> · <i>cm H₂O</i> ⁻¹
+/+	N	0.45 ± 0.057	0.039 ± 0.0012
W/W ⁿ	N	0.42 ± 0.070	0.039 ± 0.0013
+/+	ipNeb	0.40 ± 0.062	0.036 ± 0.0020
W/W ⁿ	ipNeb	0.44 ± 0.063	0.035 ± 0.0018

Lung resistance and dynamic compliance values in sensitized and challenged mice. RL and Cdyn values were obtained in the different groups of animals after sensitization and challenge but before exposure to MCh. The results for each group are expressed as means ± SEM (*n* = 8).

thing, exceeded the response in the congenic littermates, with a shift to the left in the methacholine dose-response curve for both RL and Cdyn.

Discussion

Mast cells and their released products are widely believed to contribute to the development of allergic respiratory disorders. IgE-dependent activation of mast cells can induce these cells to release a panel of preformed or newly synthesized mediators including histamine, tryptase, prostaglandins, leukotrienes, and platelet activating factor, which can result in acute phase allergic reactions in the lung including airway obstruction, airway microvascular leakage, and mucosal edema, as well as mucus gland hypersecretion (13–15). Although a role for mast cells has been defined in the acute phase of allergic reactions, much less is known about their role in chronic allergic lung inflammatory responses and their contribution to lung dysfunction in this setting. After allergen sensitization and challenge in the mouse, the changes in airway function that have generally been monitored include the response to MCh (8) or electrical field stimulation of tracheal smooth muscle preparations (16) and likely reflect a more chronic, eosinophil-dependent response (12). The current study extends previous investigations by assessing airway responsiveness *in vivo* and factors, such as cells and antibodies, which may contribute to the development of airway responsiveness.

Sensitization and challenge of the mast cell-deficient mice resulted in IgE and IgG1-specific antibody responses, increased eosinophils in the BAL and lung digests, and peribronchial infiltration of eosinophils. In all of these aspects, they were indistinguishable from their congenic littermates. The only difference was that mast cells were identified histologically in the submucosa of +/+ mice and not in the W/Wⁿ animals. These findings suggest that the development of an allergic inflammatory reaction is not dependent on the presence of functional mast cells. These results are similar to what has been suggested in other systems. For example, Nogami and coworkers (17) showed no evidence for the involvement of mast cells in the pulmonary eosino-

RL and Cdyn changes in mice

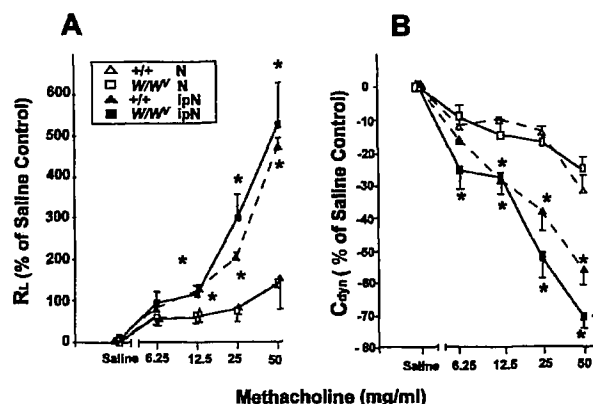


Figure 4. Lung resistance (A), and pulmonary dynamic compliance (B) in sensitized and challenged mice. RL and Cdyn values were obtained in response to increasing concentrations of methacholine as described in Materials and Methods. The results for each group are expressed as means ± SEM (*n* = 8). *Significant differences (*P* < 0.05) between the groups. *Significant differences (*P* < 0.05) between W/Wⁿ and +/+ mice.

philic response to challenge with an extract from the parasite *Ascaris suum*. Further, Brusselle et al. (18) demonstrated no effect of mast cells on eosinophil influx in BAL fluid after repeated challenge with OVA in sensitized mice. In contrast, Kung et al. (19) reported that OVA challenge of sensitized mast cell-deficient mice produced fewer eosinophils in the BAL fluid and lungs compared with similarly sensitized and challenged congenic littermates. However, in this study both the sensitization and challenge protocol were attenuated and the number of eosinophils was significantly lower than we and others (18) generally observe after sensitization and challenge as described in this study. In their protocol, mice were challenged with antigen on only 1 d; in our studies we have found that 1 or 2 d of antigen challenge were not sufficient to develop airway hyperresponsiveness (our unpublished data). In our protocol, airway inflammation and eosinophil accumulation may have been sufficiently strong so that a role for the mast cells could not be demonstrated.

The presence of a comparable peribronchial eosinophil response in the W/Wⁿ and +/+ mice was associated with a similar response to MCh in this study. Monitoring both resistance and dynamic compliance, aerosolized MCh resulted in a dose-dependent increase in RL as well as a 60–70% decrease in compliance. These changes were only observed in sensitized and challenged animals. At virtually all concentrations of MCh, the findings in the W/Wⁿ mice exceeded those in the +/+ mice. At present, there is no apparent explanation for these differences. One possibility is that in the airways of mast cell-sufficient animals, heparin is released after activation (20), and may limit the response to the cationic protein mediators released by these same or other cells. In this regard, it has been previously shown that heparin sulfate and other polyanionic molecules block the

increase in airway responsiveness caused by highly charged cationic proteins (21, 22).

If sensitization and repeated challenge with antigen triggers both eosinophilic inflammation and AHR, does this eliminate a role for mast cells in the development of these changes? It is possible that the same physiologic response, AHR to MCh challenge, may be mediated by distinct cellular mechanisms in different strains of mice (23). For example, in BALB/c mice AHR could be induced in an IgE and mast cell-dependent fashion. In strains genetically deficient in important mast cell mediators (e.g., mast cell protease 7 deficiency in C57BL/6 mice) AHR may be more dependent on other cell types, such as eosinophils. Mast cell deficient mice of the same background as studied here do demonstrate reduced severity of anaphylaxis induced by anti-IgE treatment as well as reduced airway responsiveness to MCh shortly after systemic administration of anti-IgE (25, 26).

After limited bronchoprovocation mast cells may play a role in the liberation of cytokines such as IL-4, IL-5, and TNF- α (15); because of preformed stores, mast cells could provide the initial source of TNF- α in IgE-dependent reactions (27). However, we have shown in nude mice that were passively sensitized with IgE, that despite adequate mast cell degranulation, insufficient cytokines are liberated to trigger an eosinophilic response (28). A similar inconsistency centers around the role of IgE in triggering allergic

inflammation and AHR. Sensitization exclusively via the airways, which results in limited eosinophilic infiltration of the peribronchial regions, results in AHR that appears to be IgE dependent (29). On the other hand, after sensitization and challenge as performed in this study, AHR may be IgE independent but eosinophil dependent (our unpublished data). Similar results have recently been reported by Korsgren et al. (30) demonstrating normal development of eosinophilic airway inflammation in B cell-deficient mice. However, we can not discount that in the mast cell-deficient mice, the presence of a normal IgE response serves to trigger other cells expressing either high affinity IgE receptors, e.g., basophils, macrophages, or other cells expressing low affinity receptors (6) to release important proinflammatory mediators.

In summary, in this study of mast cell-deficient mice we have shown that after sensitization and airway challenge they are capable of developing an allergic antibody response and changes in airway resistance and dynamic compliance that are similar to their congenic littermates. Although this does not exclude contributions of mast cells in other aspects of chronic allergic inflammatory responses, it indicates that mast cells do not have an essential role in development of eosinophilic airway inflammation and airway hyperresponsiveness to MCh in mice sensitized and challenged as described in this report.

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EXHIBIT D

Review

Open Access

Measuring the lung function in the mouse: the challenge of size

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Abstract

Measurement of the effects of drugs, mediators and infectious agents on various models of lung disease, as well as assessment of lung function in the intact mouse has the potential for significantly advancing our knowledge of lung disease. However, the small size of the mouse presents significant challenges for the assessment of lung function. Because of compromises made between precision and noninvasiveness, data obtained may have an uncertain bearing on the mechanical response of the lung. Nevertheless, considerable recent progress has been made in developing valid and useful measures of mouse lung function. These advances, resulting in our current ability to measure sophisticated indices of lung function in laboratory animals, are likely to lead to important insights into the mechanisms of lung disease.

Introduction

Much of our current understanding of the normal functioning of the lung and mechanisms of lung disease comes from studies utilizing animals. As one clear example, animal systems of a wide variety of species, including humans, provided the essential mechanistic proof of a link between inflammation and airways hyperresponsiveness that set the stage for current anti-inflammatory therapy [1]. Mice are now widely employed in lung research because of certain advantages this species is thought to provide [2]. Advantages of using mice include a well-understood immunologic system, the vast array of available reagents, a short reproductive cycle, a well-characterized genome, the advent of transgenic technology, and economic factors [2-4]. Using mice as models of human disease, in particular asthma, has certain shortcomings [2,5] only some of which will be covered in this review. For any animal system to yield useful and valid insights into disease it must exhibit an appropriate phenotype. It has be-

come apparent that the valid assessment of lung function in an animal as small as the mouse requires that a number of technical challenges be overcome.

The paucity of information on the measurement of lung function in the mouse has largely reflected the difficulty of measuring the necessary respiratory signals of flow, volume and transpulmonary pressure. This applies particularly to the small gas flows involved [6,7]. However, the work of Martin *et al* in 1988 demonstrated that measurements of pulmonary resistance and compliance could be made in this small species [8]. At about the same time, Levitt and Mitzner clearly illustrated the utility of using mice to explore the genetics of hyperresponsiveness [9,10]. Since these studies, the use of mice to study lung disease has increased dramatically and a number of approaches have been developed in the ensuing years for measuring lung function in mice *in vivo*. In this review we examine these various methods and discuss their

**Figure 1**

Photomicrograph of the parenchyma and respiratory bronchioles of a mouse (20 gram female BALB/c) lung. Note the rapid branching from a conducting airway into alveolar ducts and the relatively large airways. Stain is H & E with 5 × magnification.

respective attributes. Each approach represents a compromise between accuracy, non-invasiveness, and convenience [11].

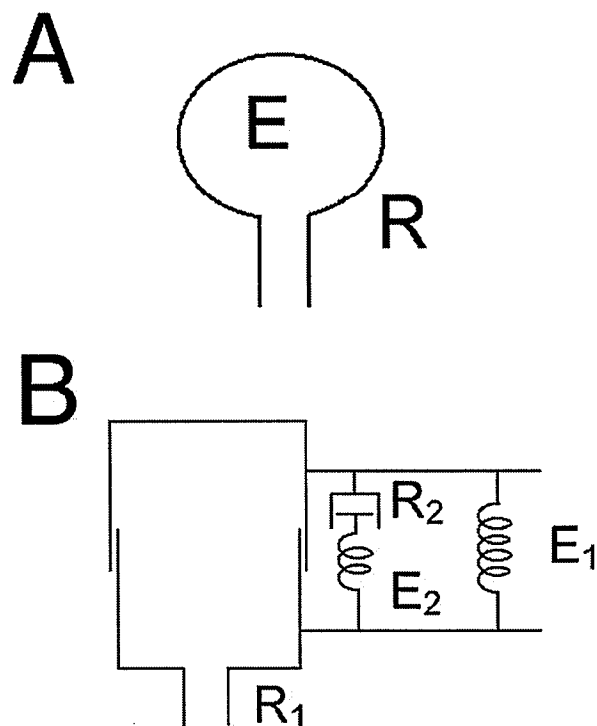
Lung anatomy

One look through the microscope at a section of mouse lung (Figure 1) demonstrates that the mouse lung is considerably different in structure from the human lung, although relatively little has been published about the architecture of the mouse lung compared to other species. What is known about the structure of the mouse lung probably has important bearing on its function [12–14]. The total lung capacity (TLC) of the mouse is about 1 ml compared to 10 ml of the rat and 6,000 ml of a human. Like the human, there are 5 lobes in the right mouse lung, but unlike the human the mouse has only a single left lung. Also unlike the human lung, but similar to the rat, the mouse pleura is thin, yet it is strong enough to be inflated to considerably higher pressures than the 30 cm H₂O normally associated with TLC (W Mitzner, personnel communications). The parenchyma of the mouse lung occupies a smaller fraction of the total lung than that of the rat but more than that of the human (mouse: 18%, rat: 24%, human: 12% lung volume). The alveoli of the mouse lung are smaller (80 μm mean linear intercept (MLI)) than those of the rat (MLI 100 μm) or human (MLI 210 μm). The blood-gas barrier thickness in the mouse (0.32 μm) is similar to that of the rat (0.38 μm) but some-

what smaller than that of the human (0.62 μm), which might have important implications for both gas exchange and parenchymal lung mechanics. The airways constitute a large percentage of the lung in mice (11%) compared to rats (5.7%). Cartilage is present in the mouse trachea but is less well organized than in other species; only the upper part of the trachea has the complete rings seen in other mammals and these rapidly change to plates as one proceeds distally. Mouse lungs have fewer respiratory bronchioles and airway generations (13–17 generations) than do human lungs (17–21 generations) with the airways of the mouse lung exhibiting a monopodial as opposed to dichotomous branching pattern. Two other significant features of the mouse lung are the thinness of the respiratory epithelium and the relatively large airway lumen [12,14]. This large airway caliber is speculated to reduce the flow-resistive load that would otherwise result from the rapid respiratory rate (250–350 bpm) required by the mouse to maintain body temperature [15]. An important functional difference between mice and rats compared to humans is the paucity, or even complete absence, of sub-mucosal glands and the presence of high numbers of Clara cells [12]. Exactly what significance all these anatomical features of the mouse lung have for lung function is speculative, but it has been our experience that the baseline airway resistance of mice that have been sensitized and challenged with antigen differs imperceptibly from that of control animals [16,17]. This suggests that inflammatory processes that could compromise lung function in larger animals (e.g. humans) might have little effect in mice because of their relatively large airway size and/or lack of mucous glands.

Basic mechanical models of the lung

Measurement of the function of the lung, especially assessment of lung mechanics, is typically done in the context of a model of the lung [18–20]. The simplest model is a tube connected to a bellows (Figure 2A). This model works well for a single breathing frequency, but has major limitations when the changes in lung mechanics that occur with alterations in breathing frequency are considered. This is because the resistive and elastic properties of the lung are substantially dependent on breathing frequency. For example, the resistance of the lung falls as frequency increases over the range associated with normal breathing [21]. To model this type of mechanical behavior, spring-and-dashpot assemblies capable of simulating viscoelastic behavior need to be included in the model (Figure 2B). These basic models allow us to develop mathematical expressions, which can be used to quantitatively assess lung mechanics. The parameters of the models, that is, the resistive and elastic values of their individual components, constitute the endpoints we use to assess lung function experimentally.

**Figure 2**

Two common and basic mechanical models of the lung. A: A homogeneously ventilated model consisting of a single elastic balloon (elastance E) served by a single flow-resistive pipe (resistance R). B: A homogeneous model again with a single airway (resistance R_1), but with a Kelvin body consisting of two springs (E_1 and E_2) and a dashpot (resistance R_2) to account for the viscoelastic behavior of the lung tissue.

The viscoelastic model in Fig. 2B does a substantially better job of describing frequency-dependent nature lung mechanics than the model in Fig. 2A. Nevertheless, the simple model in Fig. 2A still serves as the conceptual platform for most studies of lung mechanics and bronchial responsiveness. The mechanical behavior of this model is described by its equation of motion. This equation is based on simple physics and states that the force (pressure) applied to the model is equal and opposite to the opposing force (pressure) the model generates. The applied pressure, P , is that supplied either by the respiratory muscles or a mechanical ventilator. The opposing pressure is made up, in general, of three components: a resistive pressure (P_{res}), an elastic pressure (P_{el}), and an inertive pressure (P_{in}).

Thus,

$$P = P_{res} + P_{el} + P_{in} \quad (\text{Equation 1})$$

P_{res} is described by Ohm's law:

$$P_{res} = R\dot{V} \quad (\text{Equation 2})$$

where R is the resistance of the lung and \dot{V} is flow of gas. P_{el} is described by Hooke's law:

$$P_{el} = EV = \frac{1}{C}V \quad (\text{Equation 3})$$

where E is lung elastance (equal to the inverse of compliance, C) and V is lung volume relative to functional residual capacity. P_{in} comes into play only at frequencies well above those of normal breathing, while both P_{res} and P_{in} become negligible when frequency is extremely low. Thus, the equation of motion relevant to normal breathing is

$$P = R\dot{V} + \frac{1}{C}V \quad (\text{Equation 4})$$

The parameters R and E are both profoundly dependent on breathing frequency and lung volume.

Lung volume

The volume of the lungs has an important influence on its pressure-flow relationships. For example, an increase in lung volume stretches the airways open and so causes airway resistance to fall (tethering). This also makes it more difficult for the airways to narrow when the airway smooth muscle contracts, and represents an important mechanism by which the challenged lung can defend airway caliber [18,22]. Unlike larger laboratory animals, the measurement of lung volume in the mouse is particularly problematic due to its small size. For example, when thoracic gas volume is measured using the conventional Boyle's Law technique, the volumes of air in the transducers used to measure plethysmographic and airway-opening pressures must be small relative to the lung volume, or significant measurement errors will occur. It has only recently been reported that measurement of functional residual capacity (FRC) by this approach is at all feasible [23]. The measurement of FRC with gas dilution is equally difficult, again due to the small size of the mouse lung, and there are only a few reports in the literature on the use of this technique [24]. Other studies of mouse lung volume have used a buoyancy approach [25], a degassing approach [26,27], and even a CT scanner method has been reported [28]. None of these, however, is particularly practical for most study designs. Better techniques for measuring lung volumes in mice are certainly needed, so this will be a fruitful area for future research.

Lung elastance (compliance)

The component of the transpulmonary pressure loss that is out of phase with flow and in phase with volume, as well as the recoil pressure exerted by the lung under static conditions, are caused by the elastic forces within the lung. The loss of elastic recoil within the lung defines emphysema while an increase defines restrictive processes [18,25]. The chest walls and other thoracic structures in mice are extremely compliant, so most elastic recoil measured in an intact animal can be attributed specifically to the lung. Moreover, the elastic recoil of the lung shows considerable genetic variability that needs to be taken into account in study designs [26]. The elastic recoil of the lung is conveniently assessed in terms of the quasi-static pressure-volume (PV) curve measured by inflating and deflating the lung in a step-wise fashion. The inspiratory limb of the curve traverses a path through values of P that are higher than those of the expiratory limb, the difference between the two limbs being termed hysteresis. Changes in the inspiratory limb of the PV curve that cause an increase in hysteresis are taken to indicate enhanced airway closure, such as that observed in humans after dry cold gas inhalation [29] and recapitulated in mice with allergic inflammation [30]. These changes in PV characteristics can be sensitive indicators of lung dysfunction and contribute to the genesis of hyperresponsiveness. The shape of the pressure volume relationship is one manifestation of the nonlinear characteristics of lung mechanics in the normal, unperturbed lung. Airflow resistance also exhibits alinear behavior as the airflow reaches high rates of flow as sudden changes in luminal dimensions occur (e.g. vocal chords). The mouse lung exhibits alinear elastic (compliance) behavior that increases following antigen challenge, a change that is most consistent with reopening airways that were closed [29,30]. Airflow is not alinear (i.e. laminar flow regimes) in either condition as it is highly unlikely turbulent flow occurs in mouse lungs due to the small airway diameters, unlike humans where turbulent flow is a common occurrence [30], pointing to a clear limitation of this species in exploring complex airflow conditions.

Phenotyping uncertainty principle

Accurate and valid measurement of lung mechanics in laboratory animals is a balancing act between measurement precision and maintenance of "natural" conditions. This situation is similar to the Heidelberg uncertainty principle of quantum mechanics which states that the measurement of a particle's position interferes with the measurement of its movement, and *vice versa* [31]. In a similar fashion, as we make more precise measurements of lung function in an animal, we are forced to constrain the animal's behavior in a way that departs from the maintenance of natural conditions [11]. At the extreme ends of this continuum are the measurements derived from the free roaming animal in a closed chamber, known

Phenotyping Uncertainty Principle

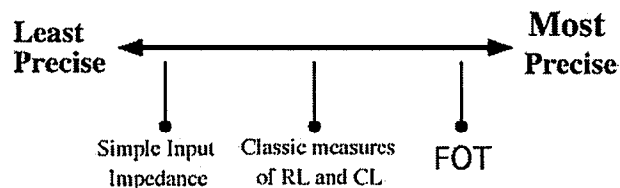


Figure 3

The non-invasiveness-precision continuum of the phenotyping uncertainty principle – see text for discussion.

as unrestrained plethysmography (UP), and the measurement of input impedance using forced oscillations performed in an anesthetized, tracheostomized animal (Figure 3).

Unrestrained plethysmography

This approach to assess lung function involves placing the subject into a small closed box and measuring the pressure changes within the box that occur as the animal breathes [7,11,32]. The animal is conscious and unrestrained. This technique currently enjoys wide popularity (for example see [33]) because 1) it is simple and 2) the mouse remains unharmed after the experiment. The endpoint is the heuristic variable known as Penh, which stands for 'enhanced pause'. It is important to note that there is no linkage between Penh and other variables that are derived from mechanical principles – Penh is merely an empirical derivative of the respiratory variations in box pressure [11]. While an earlier publication demonstrated reasonable correlations between Penh and invasive measures of lung mechanics [32], recent publications draw into serious question the validity of using Penh to measure lung function [7,11,34].

The pressure changes occurring within the box as the mouse breathes are derived first from gas compression and decompression within the thorax – an event linked to the state of lung mechanics – and second from humidification and warming of inspired gas – an event unrelated to lung mechanics. During bronchoconstriction, both components increase [7], but much of this increase is likely due to the increased stimulation to breathe that would arise from chemoreceptor receptors in the lung. Hence box pressure changes should be influenced by chemoreceptor sensitivity and genetics that control responses to chemo- or irritant- receptor stimulation and integration [11,35]. Recent studies show that

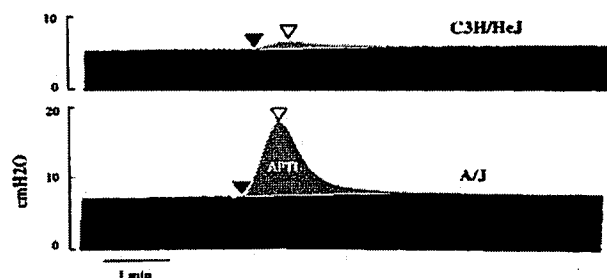


Figure 4

Pulmonary impedance measurements in anesthetized mice. The diagram shows airway opening pressure plotted against time. Volume and flow excursions for each breath are maintained constant by use of a volume-cycled ventilator. Increases in the magnitude of lung impedance following acetylcholine injection are assessed as the increase in pressure above baseline. Note the increased responsiveness in the A/J compared the C3H/HeJ strain of mouse. APTI: Airway pressure time index. Used with permission [38].

changes in P_{enh} depart from mechanical changes during a state of increased box temperature [7,34] in an exactly opposite way during exposure to hyperoxic conditions [34,35] and temporarily [36]. These findings show that P_{enh} is not a valid measurement of the lung function of the mouse except as a measure of patterns of respiration, and it has been known for a long time that patterns of respiration usually have little bearing on lung mechanics. Finally, a response in P_{enh} may also be due to changes in nasal cavity resistance, as the upper airways are very significant contributors (50%) to total lung resistance and their contribution is likely to change depending on the experimental situation [32].

Lung impedance magnitude

The next step on the phenotyping uncertainty continuum (Figure 3) is the measurement of the magnitude of respiratory system or lung impedance. Lung impedance is a complex quantity having both real and imaginary parts (see section 'Forced oscillations and the constant phase model'), and its calculation requires rather sophisticated methods. The magnitude of impedance ($|Z_{rs}|$), however, is easily determined simply as the ratio of the absolute value of the swing in pressure (ΔP) to the absolute value of the swing in flow ($\Delta \dot{V}$) occurring over a breath, thus

$$|Z_{rs}| = \frac{|\Delta P|}{|\Delta \dot{V}|} \quad (\text{Equation 5})$$

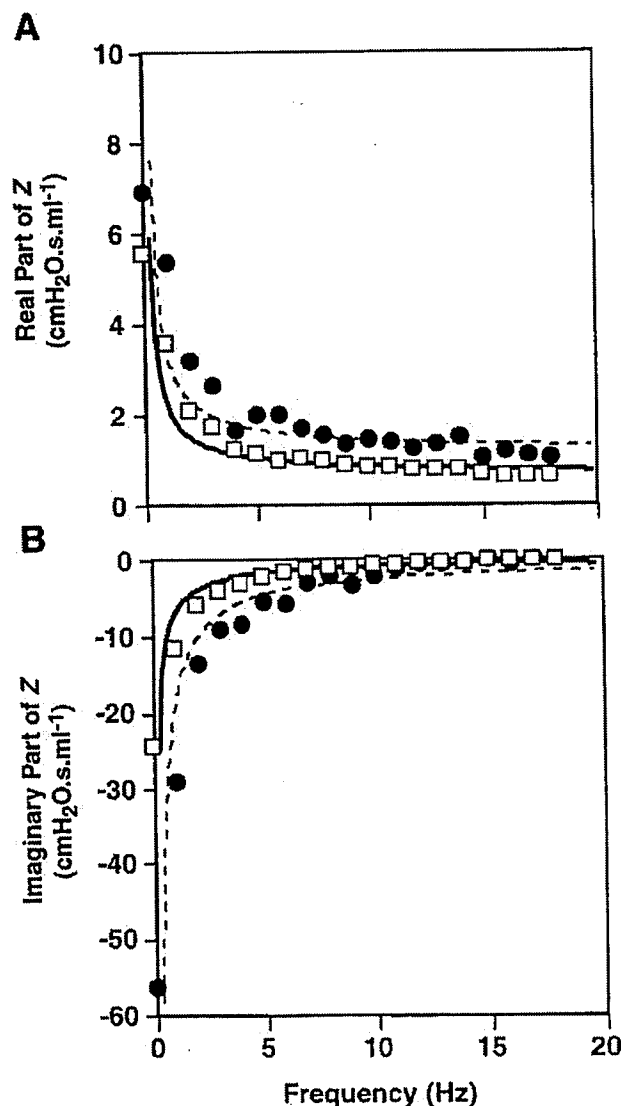
As seen in the example in Figure 4, pressure is obtained by placing a pressure transducer at the airway opening, while flow is assumed to be constant as the animal is mechanically ventilated with a volume-cycled ventilator [9,10,37,38]. When a bronchoactive agent is introduced, the peak pressure with each breath goes up, so $|Z_{rs}|$ increases commensurately. Hence, by merely measuring airway-opening pressure, a useful index of lung function is derived. This technique has been used because it is simple and gives a direct assessment of lung mechanics [9,10,37,38].

The major disadvantage of this technique is that even though a direct measure of lung function is made, no insight is obtained as to where in the lung an abnormality might be located. This is a significant limitation if one wishes to explore the mechanisms of bronchoconstriction and whether it reflects, for example, central versus peripheral airways dysfunction. Nevertheless, this simple approach has produced significant advances in our understanding of the genetics of hyperresponsiveness [3,9,10].

Measurement of dynamic resistance (R_L) and compliance (C_L)

A classic approach to assessment of lung mechanics in animals is the measurement of dynamic lung resistance (R_L) and compliance (C_{dyn} or C_L) [3,8,20,38–40]. In the past, this approach was often used to assess central versus peripheral alterations in lung mechanics – a topic of considerable current interest. The calculation of R_L and C_{dyn} requires the measurement of intrathoracic pressure that, in larger animals, is obtained with an esophageal balloon or pleural catheter, but in a mouse is obtained either by opening the chest or by making the reasonable assumption that the chest wall presents little mechanical load compared to that of the lung [26,41,42]. Flow is usually obtained with a pressure transducer but this approach is problematic when miniaturized to the mouse [7,43]. Accordingly, flow is commonly derived from the differentiation of a volume signal, usually obtained from a body plethysmograph [8,40]. The values of R_L and C_L are then derived by fitting the equation of motion (Equation 4) to measurements of pressure, flow and volume.

The measurement of R_L and C_L , while technically challenging, does yield additional insight into the mechanisms of bronchoconstriction over that provided by $|Z_{rs}|$. Generally speaking, an increase in R_L reflects both narrowing of the conducting airways and alterations in the lung periphery (heterogeneous narrowing or closure of distal airways together with changes in the intrinsic mechanical properties of the parenchyma). Decreases in C_L , on the other hand, reflect only events in the lung periphery, particularly airway closure leading to lung unit derecruitment [44]. If the response to an intervention is limited largely

**Figure 5**

The respiratory input impedance of the mouse. Open squares represent baseline conditions while closed circles show the result of administering an aerosol of methacholine. The solid and dashed lines are the fit provided by the constant-phase model (Eq. 6). Used with permission [17].

to R_L , then a relatively proximal location is implicated for the effect. By contrast, a selective change in C_L is indicative of a more distal site of action [3,8,45]. As an example of this approach, R_L and C_L were clearly shown to be independent variables in mice treated with an antibody agonist for VLA-4, an adhesion protein of the eosinophil [45]. Furthermore, the genetic dependence of these variables

suggests that the factors that control central airway function (reflected in R_L) are different from those that control peripheral airway function (reflected in C_L) [46].

Forced oscillations and the constant phase model

At the far end of the phenotyping uncertainty principle lies the forced oscillation technique (FOT) applied in anesthetized, paralyzed, tracheostomized animals to measure the complex input impedance (Z_{rs}) of the lungs [21]. We have already covered the concept that the magnitude of Z_{rs} ($|Z_{rs}|$) is a generalization of the changes of resistance and compliance, and that Z_{rs} consists of two parts that are both functions of frequency. The real part of Z_{rs} is directly related to the resistance and provides essentially the resistance of the respiratory system at the frequency in question. The imaginary part of Z_{rs} is called the reactance and reflects respiratory compliance at frequencies below 20 Hz in the mouse. Thus, the FOT essentially provides R_L and C_L at each frequency contained in the flow signal applied to the lungs. This requires that the animal be oscillated with a complex flow wave produced by either a loudspeaker [34,47,48] or a computer-controlled piston [6,7,17]. The data of pressure and either flow or volume are converted into the frequency domain by the Fast Fourier transform, and their ratios calculated to yield the real and imaginary parts of Z_{rs} (Figure 5).

The key advantage of this approach, as compared to the determination of R_L and C_L or $|Z_{rs}|$, is that Z_{rs} can be fitted to a more complex model of the lung known as the constant-phase model [49] which makes a clearer distinction between central and peripheral events in the lung. The equation of motion of the constant-phase model is

$$Z_{in}(f) = R_{aw} + i2\pi f I_{aw} + \frac{G_{ti} - iH_{ti}}{(2\pi f)^\alpha} \quad (6)$$

where R_{aw} is the resistance of the airways that are attached to the constant phase element, I_{aw} is the inertance of the gas in the airways (which has negligible effect in the mouse below 20 Hz and can be ignored [17]), G_{ti} is tissue resistance or damping, H_{ti} is tissue elasticity, and i is $\sqrt{-1}$. As R_{aw} is a measure of central airways resistance, it would be expected to change if the airways are significantly narrowed. By contrast, G_{ti} reflects either changes in tissue physical properties or regional airways heterogeneity. If changes in R_{aw} are small, then any changes in G_{ti} most likely represent changes in the parenchyma or very small airways. Acute changes in H_{ti} are likely to reflect lung derecruitment (airway closure) [44], whereas chronic changes in H_{ti} would be expected to reflect changes in the intrinsic mechanical properties of the parenchyma. This technique

is now being successfully and extensively used to assess lung mechanics of the mouse [17,33,50].

Tomioka et al [17] showed that Z_{rs} can be used to track changes obtained with an even more invasive technique [51,52] – the alveolar capsule – where resistance is partitioned into a central airway and a parenchymal tissue resistance component through the direct measurement of alveolar pressure (Figure 6). In this study, the disparate behaviours of R_{aw} , G_{ti} and H_{ti} clearly show that these three quantities are independent parameters that access different aspects of lung function. For example, antigen exposure followed by methacholine challenge caused an enhancement of both G_{ti} and H_{ti} – measures of peripheral lung function – however, changes in R_{aw} , reflecting central airways, were not significantly altered. The enhanced changes in G or H in this acute state may reflect three different mechanisms: derecruitment of lung units as airways close, temporal shifts of tissue movement, and inhomogeneities of airflow distribution. Moreover these data point away from significant alterations in airway smooth muscle function and more towards enhanced or altered secretions that cause dysfunction in small airways. Interestingly, antigen challenge in either sensitized or unimmunized mice caused no significant changes in any of the parameters at baseline before methacholine challenge, likely due to the unique architecture of the mouse lung (see above).

We believe that the well-founded theoretical basis of the FOT, and its rigorous application in mice, will lead to considerable insight into the functioning of mouse models of lung disease.

Conclusion

Measurement of lung function in a creature as small as the mouse presents considerable technical challenges. However, with the exception of the measurement of absolute lung volume and the analysis of blood gases, we have now conquered the challenge of miniaturizing the instrumentation necessary for mouse lung function assessment. Application of advanced techniques such as the FOT coupled with constant-phase model analysis hold particular promise for improved characterization of lung responses to intervention and pathology. With these approaches, we can now unravel the mechanisms of airways dysfunction, the influence of genetics and the immunological factors that define the physiome of the mouse.

Abbreviations

bpm breaths per minute

C_{dyn} dynamic compliance

C_L lung compliance

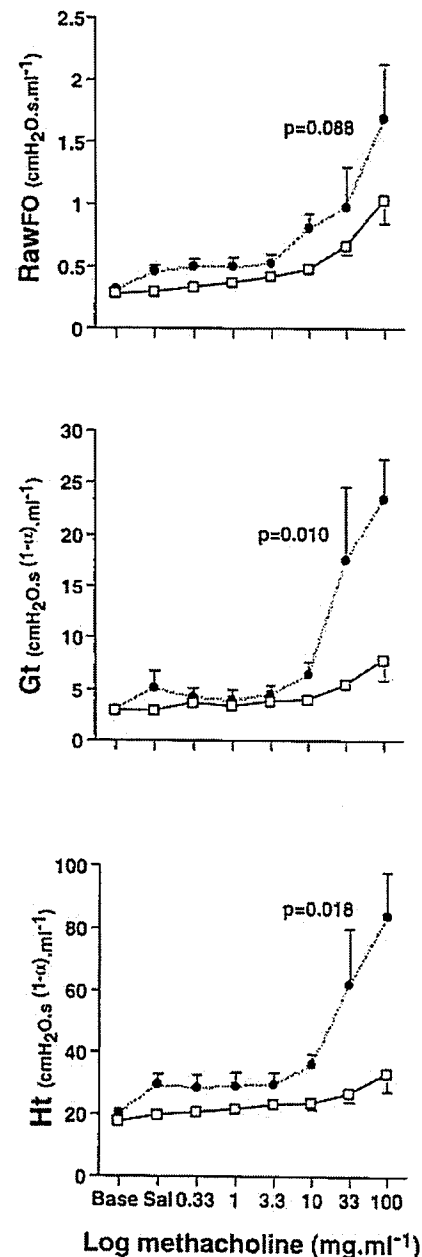


Figure 6

The mechanical response of the mouse lung to methacholine in terms of the parameters of the constant-phase model (see Eq. 6 in text). The open squares correspond to control BALB/c mice, while the closed circles represent mice sensitized to and challenged with ovalbumin. Taken from [17] with permission.

E elastance

FOT forced oscillation technique

FRC functional residual capacity

G tissue damping or tissue resistance

H elasticity

MLI mean linear intercept

P pressure

P_{res} resistive pressure

P_{el} elastic pressure

P_{in} inertial pressure

Penh enhanced pause

R_L resistance

TLC total lung capacity

UP unrestrained plethysmography

\dot{V} Flow

Z_{RS} or Z impedance of the respiratory system

$|Z_{rs}|$ Magnitude of impedance

Acknowledgements

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APPENDIX G
Kanehiro Declaration

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

GELFAND et al.

Serial No.: 09/809,753

Filed: March 14, 2001

Atty. File No.: 2879-74

For: "METHOD FOR REDUCING
ALLERGEN-INDUCED AIRWAY
HYPERRESPONSIVENESS"

Group Art Unit: 1644

Examiner: Huynh, Phuong N.

Confirmation No.: 5001

DECLARATION OF
ARIHIKO KANEHIROCommissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

U.S. Patent Application Serial No. 09/809,753

Dear Sir:

I, Arihiko Kanehiro, declare as follows.

1. I am currently an Associate Professor in the Department of Hematology, Oncology and Respiratory Medicine at the Okayama University Hospital, Okayama University Graduate School of Medicine and Dentistry, in Japan. I have an M.D. and a Ph.D.. From about September 1, 1997, to about March 31, 2000, I was employed as a Research Associate in the laboratory of Dr. Erwin Gelfand at National Jewish Medical and Research Center in Denver, Colorado. I am familiar with the research that was being conducted by Dr. Gelfand and Dr. Azzeddine Dakhama, who was working in Dr. Gelfand's laboratory as a Research Associate for part of the period that I was in the laboratory.

2. I am not an inventor of the following subject matter:

A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);

wherein said mammal has allergen-induced airway hyperresponsiveness (AHR), and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.

3. I have reviewed the pages of the documents attached as "Exhibit A." I was in the laboratory of Dr. Erwin Gelfand working as a Research Associate along with my colleague, Dr. Azzeddine Dakhama, when the work described in Exhibit A was performed. Furthermore, I confirm that I performed some of the work described in Exhibit A. Specifically, I performed a later part of the experiment in the presence of Dr. Azzeddine Dakhama after Dr. Dakhama had performed an earlier part of the experiment.

4. The header in bold on page 1 of Exhibit A includes "Azd/Ari." The letters "Azd" refers to Dr. Dakhama and "Ari" refers to my nickname. On information and belief,

U.S. Patent Application Serial No. 09/809,753

pages 1 and 2 of Exhibit A are a printout from a computer file recording the experiment. I have been informed that page 3 of Exhibit A is an image of the computer information for that file. Page 3 shows that the file was created on August 25, 1999, and it was last modified on August 27, 1999.

5. Pages 4 to 7 of Exhibit A are photocopies of my handwritten notebook pages, which include the raw data of lung function measurements I recorded for the experiment. I dated the first two handwritten pages "8/25", meaning that I performed the measurements of lung function for the experiment on a first set of animals (numbered 1 to 7) and recorded the results on those pages on August 25, 1999. The third and fourth handwritten pages are dated "8/26", meaning that I performed the measurements of lung function for the experiment on another set of animals (numbered 1 to 7) and recorded the results on those pages on August 26, 1999. I know that the dates are August 25 and 26, 1999, because the experiment is labeled "Experiment 23JL99," which meant that the experiment was initiated on July 23, 1999, and the sensitization and challenge of the mice, described on page 1 of Exhibit A, takes at least 30 days, placing the later part of the experiment that I performed toward the end of August 1999. Moreover, the computer record of the creation of pages 1-2 of Exhibit A shows the creation date as August 25, 1999, with the date last modified as August 27, 1999, one day after the conclusion of the experiment as recorded in my handwriting on pages 6-7 of Exhibit A. I also wrote "with Azd" on each of the first pages for August 25 (page 4) and August 26 (page 6), further confirming that this was the experiment with which I assisted Dr. Dakhama and which correlates with pages 1-2 of Exhibit A. Finally, the data that is typed onto pages 1-2 is clearly the same data that I recorded by hand on August 25-26. While I was conducting my part of the experiment, Dr. Dakhama was in the same room preparing the mouse lungs for further evaluation after my part of the experiment was completed. Once I

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was finished with a mouse, I would remove the mouse from the chamber of the physiology equipment and hand it to Dr. Dakhama for the further evaluation.

6. By August 1999, I was very familiar with the purpose of the experiment, and the experimental protocol, described in Exhibit A. I also consider that I was very skilled in the art in this field at that time. As noted in the "Objective" and "Hypothesis" on page 1 of Exhibit A, the purpose of the experiment was to study whether calcitonin gene related peptide (CGRP) would inhibit methacholine (MCh) induced airway hyperresponsiveness (AHR) in mice sensitized and exposed to the allergen ovalbumin (OVA), which was a mouse model of asthma. The abbreviations (CGRP), (MCh), (AHR), and (OVA) were commonly used in Dr. Gelfand's laboratory and by scientists at that time as shorthand for the terms calcitonin gene related peptide, methacholine, airway hyperresponsiveness, and ovalbumin, respectively.

7. This mouse model was used extensively in Dr. Gelfand's laboratory in 1999, and was known by those skilled in this field as an acceptable model for testing allergen-induced AHR. The following two exemplary articles discuss this mouse model: Hamelmann et al., "Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography," *Am. Respir. Crit. Care*, 156:766-775 (1997) (attached as Exhibit B); and Takeda et al., "Development of Eosinophilic Airway Inflammation and Airway Hyperresponsiveness in Mast Cell-deficient Mice," *J. Exp. Med.*, 186:449-454 (1997) (attached as Exhibit C). Those documents describe the mouse model in detail and provide experimental data showing that the model is a valid model for allergen-induced AHR, which models AHR *in vivo* in humans suffering from bronchial asthma. Hamelmann et al. and Takeda et al. show that the mouse model shares many characteristics with human respiratory conditions associated with allergic inflammation, including an IgE-associated immune response, a dependence on a Th2-type response, an eosinophil response, and both a marked and evolving hyperresponsiveness of the airways. See Hamelmann et al. at page 766,

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paragraph bridging the first and second columns; at page 770, Tables 1 and 2; at page 770, Figure 4; at page 773, Fig. 8; and at page 774, second column, last paragraph; and Takeda et al. at page 450, Figures 1 and 2; at page 450, Figure 4; and at page 452, first column, first full paragraph.

8. The mouse model uses ovalbumin as an experimental allergen. Ovalbumin was commonly referred to by the Gelfand laboratory and by the art in general as "OVA" or "Ova." Hamelmann et al. and Takeda et al., confirm this abbreviation. In an experiment using this mouse model, the mice are first "sensitized" (made sensitive or reactive) to the allergen (OVA) by repeated, systemic (intraperitoneal injection, abbreviated "i.p." or "ip") exposure to the OVA allergen. See Hamelmann et al. at page 767 "Sensitization and Airway Challenge", and Takeda et al. at page 449 "Sensitization and Airway Challenge". Exhibit A discusses this sensitization in the experiment performed by Dr. Dakhama as follows: "Balb/c mice were sensitized to OVA (2 i.p.) on day 0 and day 14." See Exhibit A at page 1.

9. This systemic sensitization with OVA is then followed by repeated airway challenge with the same allergen (OVA) in aerosol form (e.g., nebulized administration, which was referred to by the Gelfand laboratory in shorthand as "N" or "Neb"). See Hamelmann et al. at page 767 "Sensitization and Airway Challenge", and Takeda et al. at page 449 "Sensitization and Airway Challenge". Exhibit A discusses this challenge with OVA in the experiment performed by Dr. Dakhama as follows: "On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." See Exhibit A at page 1.

10. Subsequent to the aerosol challenge with OVA, the mice are subjected to increasing doses of aerosolized methacholine (MCh). See Hamelmann et al. at page 768, col. 2, first full paragraph, and Takeda et al. at paragraphs spanning pages 449-450 under "Determination of Airway Responsiveness"; and at page 452, Figure 4. It was known that in

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such a model, after exposure to the provoking agent MCh, mice that have been sensitized to OVA and subsequently challenged with OVA show increased AHR compared to control mice that have not been both sensitized and challenged with the allergen OVA. See Takeda et al. at page 451, second column, second paragraph, and page 452, second column, lines 3-7 of second paragraph. Thus, it is clear that in the model the AHR is allergen-induced.

11. In 1999, there were different acceptable methods for measuring AHR. Hamelmann et al. discusses a noninvasive method, and Takeda et al. discusses an invasive method. I was very experienced in the invasive method, which I used in the work described in Exhibit A. After Dr. Dakhama sensitized and challenged the mice with OVA as described on page 1 of Exhibit A, he gave the mice to me. In August 1999, I practiced an invasive method for measuring AHR in the mouse model similar to the method described in Takeda et al. at page 449, second column, to page 450, first column. I used that method in the work described in Exhibit A. In that method, I anesthetized and tracheostomized the mice. I then attached a connector to the tracheostomy tube, with ports connected to the inspiratory and expiratory sides of two ventilators. One ventilator was used for mechanical ventilation of the lung and the other was used to administer the MCh into the lung. (In the method discussed in Takeda et al., only one ventilator was used.) I connected another port of the connector to a pressure transducer, which was in turn connected to a plethysmograph.

12. The mice were then challenged with increasing doses of MCh. See Takeda et al. at paragraphs spanning pages 449-450 under "Determination of Airway Responsiveness"; and at page 452, Figure 4. After each dose of MCh, transpulmonary pressure was detected with the pressure transducer. *Id.* at page 450, first column. Also, "[c]hanges in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer" *Id.* Also, "[f]low was measured by digital differentiation of the volume signal." *Id.* "Lung resistance (R_L) and

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dynamic compliance (C_{dyn}) were continuously computed . . . by fitting flow, volume, and pressure to an equation of motion.” *Id.* “Maximum values of R_L and minimum values of C_{dyn} were taken to express changes in murine airway function.” *Id.* As discussed above, this is the method I used in the work described in Exhibit A. The increasing doses that I used in the work described in Exhibit A are shown in the left hand column on pages 4 to 7 of Exhibit A as “1.56,” “3,” “6.25,” and “12.5.” Those doses are also shown in left hand column of the copy of the data at pages 1 and 2 of Exhibit A.

13. In general, one skilled in the relevant art in August 1999 understood that an increase in R_L reflects both narrowing of the conducting airways and alterations in the lung periphery, while decreases in C_{dyn} reflect events in the lung periphery (Reviewed in Irvin and Bates, *Respiratory Research*, 4:1-9 (2003) (attached as Exhibit D), at page 5, col. 2). Increases in R_L and decreases in C_{dyn} correlate with increased AHR. See Takeda et al., at page 451, last four lines of col. 2; Figure 4.

14. We used this mouse model in Dr. Gelfand’s laboratory to test for the effect of treatment with a test agent on AHR. Mice that were sensitized and challenged with OVA, but which did not receive the experimental treatment, served as a positive control. A negative control for the experiment was generated by exposing one group of mice to PBS (a buffer) instead of OVA during the sensitization and nebulization challenge period. Alternatively, negative control mice could be exposed to PBS during the sensitization period and then exposed to the nebulized OVA during the challenge period, or vice versa (OVA sensitization and PBS challenge). AHR was then measured in the mice after inducing airway responses (bronchoconstriction) by exposing the mice to a provoking agent (stimulus), such as MCh. Negative control mice would not show significant AHR in response to the provoking stimulus MCh. The positive control mice that have been both sensitized to, and challenged with, OVA would respond to MCh exposure with a significant AHR compared to

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the negative control mice, both in terms of the dose of MCh required to increase AHR (a lower dose will result in more AHR), and in the elevation of the maximal response to MCh.

15. By comparing the AHR of the treated mice to the AHR of the positive and negative control mice, we could determine if administration of the test agent resulted in inhibition of allergen-induced AHR compared to mice that did not receive treatment with the test agent. In August 1999, this was an accepted method of testing whether a test agent inhibited allergen-induced AHR in mammals compared to mammals that did not receive treatment with the test agent.

16. Exhibit A shows the use of this method to test whether administration of the test agent CGRP would inhibit allergen-induced AHR in the mice sensitized to, and challenged with, OVA, compared to mice that were not administered CGRP. Exhibit A describes four groups of mice that Dr. Dakhama prepared.

17. The first group had four mice that were prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. The mice in the first group were then pretreated with an i.p. injection of CGRP (200 μ l of 10^{-6} M) two hours prior to MCh exposure. Exhibit A at page 1. The results I obtained with the mice in this first group are shown at pages 4 and 5 of Exhibit A with the term "IPN + CGRP," mice (1), (2), (3), and (4). The same data is reproduced on page 1 of Exhibit A under the heading "Group 1: OVA ip/Neb + CGRP." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, I used the abbreviation "IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. Page 1 of Exhibit A uses the term "OVA ip/Neb" for the same

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mice. The "+ CGRP" in the terms at pages 1 and 4 indicates that the mice were also administered CGRP.

18. The second group had three mice that were prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. The mice in the second group were then pretreated with an "i.p. injection of CGRP antagonist (100 μ l of 10^{-5} M) followed by injection of CGRP (200 μ l of 10^{-6} M)" two hours prior to MCh exposure. Exhibit A at page 1. The results I obtained with the mice in this second group are shown at page 5 of Exhibit A with the term "IPN + α -mAb + CGRP," mice (5), (6), and (7). The same data is reproduced on page 1 of Exhibit A under the heading "Group 2: OVA ip/Neb + Antag + CGRP." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, I used the abbreviation "IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. Page 1 of Exhibit A uses the term "OVA ip/Neb" for the same mice. The terms "+ α -mAb" and "+ Antag" refer to the CGRP antagonist. The "+ CGRP" in the terms at pages 1 and 4 indicates that the mice were also administered CGRP.

19. The third group had three mice that were prepared as follows. The mice "were not sensitized and not challenged [with OVA]." These were negative control mice which also were not administered CGRP. Exhibit A at page 1. The results I obtained with the mice in this third group are shown at page 6 of Exhibit A with the term "Saline Neb", mice (1), (2), and (3). The same data is reproduced on page 1 of Exhibit A under the heading "Group 3: PBS ip/Neb untreated (-CTL)." The terms "Saline Neb" and "PBS ip/Neb" indicates that the mice were neither sensitized to, nor challenged with, OVA. The term that I used "Saline Neb" did not include the term "+ CGRP," which indicates that the mice were not

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administered CGRP. This same information is conveyed in the Group 3 designation at page 1 of Exhibit A with the term "untreated (-CTL)."

20. The fourth group had three mice that were prepared as follows. The mice "were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days." Exhibit A at page 1. These mice were positive control mice which were not administered CGRP. Exhibit A at page 1. The results I obtained with the mice in this fourth group are shown at page 7 of Exhibit A with the term "OVA IPN," mice (4), (5), (6), and (7). The same data is reproduced on pages 1 and 2 of Exhibit A under the heading "Group 4: OVA ip/Neb untreated (-CTL)." As discussed above, the "IP" or "ip" is an abbreviation for the intraperitoneal injection with an agent, and the "N" or "Neb" refers to the exposure to an aerosol form of an agent (nebulized administration). At page 4 of Exhibit A, I used the abbreviation "OVA IPN" for mice that had been sensitized to OVA by injection and challenged with OVA in aerosol form. Page 1 of Exhibit A uses the term "OVA ip/Neb" for the same mice. The term that I used "OVA IPN" did not include the term "+ CGRP," which indicates that the mice were not administered CGRP. This same information is conveyed in the Group 4 designation at page 1 of Exhibit A with the term "untreated (+CTL)."

21. Dr. Dakhama provided the mice from Groups 1 to 4 to me so that I could expose the mice to the MCh and make the measurements of lung resistance (R_L) and dynamic compliance (C_{dyn}) as discussed above. I now discuss my handwritten data on pages 4-7 of Exhibit A, which have been copied into the tables on pages 1-2 of Exhibit A. For each mouse, I recorded the body weight of the mouse (BW). I then recorded for each mouse, two values for each of a baseline (BL) (prior to administration of MCh), saline administration (SAL) (0 mg/ml MCh), and then doubling doses of MCh from 1.56 mg/ml to 12.5 mg/ml. Referring to the two values recorded at each data point, the first number is the value for R_L .

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and the second number is the value for C_{dyn} . The number that I recorded for C_{dyn} is written in shorthand. The actual number was 10^{-3} of the recorded number.

22. On August 25, 1999 (8/25), as shown on pages 4-5 of Exhibit A, we evaluated the first seven mice, and on August 26, 1999 (8/26), as shown on pages 6-7 of Exhibit A, we evaluated the last seven mice. The evaluations had to be split over two days because evaluation of 14 mice would have required too much time for one day. As shown on page 4, I evaluated the first group of mice discussed above, which I designated "IPN + CGRP." As discussed above, that was my shorthand for referring to "OVA ipNeb + CGRP" mice (allergen-sensitized and challenged) that also received the CGRP. There were four mice in that group, designated by numbers. I also evaluated the three mice in the second group of mice discussed above (CGRP + CGRP antagonist group) as shown on page 5, which I referred to as "IPN + α -mAB + CGRP," corresponding to "OVA ipNeb + Antag + CGRP" mice (allergen-sensitized and challenged) that also received the CGRP and CGRP antagonist. As shown on page 6, I evaluated the three mice in the third group of mice discussed above (the negative control group), which I designated as "Saline Neb," corresponding to "PBS ip/Neb untreated (-CTL)" on page 1 of Exhibit A. As shown on page 7, I evaluated the four mice of the fourth group of mice discussed above (the positive control group), which I designated "OVA IPN," corresponding to "OVA ip/Neb untreated (+CTL)" on pages 1-2 of Exhibit A. Mouse 6 on page 5 and Mouse 7 on page 8 both died during the procedure, which can happen. This explains the lack of data and a handwritten line in these columns.

23. These data were evaluated by comparing the changes in R_L in response to increasing doses of MCh, represented by the first number recorded for each mouse, in the presence and absence of CGRP. Changes in C_{dyn} were also evaluated by comparing the change in this number in response to increasing doses of MCh, represented by the second number recorded for each mouse, in the presence and absence of CGRP. Visual inspection of

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the raw data showed that if the positive and negative control mice are compared, the R_L values for the positive control mice appeared to be significantly higher in response to increasing doses of MCh as compared to the R_L values for the negative control mice. Compare, for example, the R_L values for mouse 1 in Group 3 (mouse 1 in the 8/26 data) to mouse 1 in Group 4 (mouse 4 in the 8/26 data). With respect to C_{dyn} , C_{dyn} values for the positive control mice appeared to be significantly lower in response to increasing doses of MCh as compared to the C_{dyn} values for the negative control mice, which was expected. With regard to the CGRP treated mice (Group I), viewing, for example, the R_L values for mouse 1 of Group 1 (mouse 1 in the 8/25 data), these values appeared to be more similar to the negative control mice than the positive control mice, as did the C_{dyn} values, indicating that CGRP inhibited AHR in the mice. In contrast, viewing, for example, the R_L values for mouse 5 of Group 2 (mouse 5 in the 8/25 data), which represented CGRP antagonist mice, these values appeared to be more similar to the positive control mice than the negative control mice, as did the C_{dyn} values, indicating that the inhibitory effects of CGRP were abolished in the presence of a CGRP antagonist. Therefore, I concluded from looking at the raw data for this experiment that CGRP inhibited AHR in allergen-sensitized and challenged mice as compared to in the absence of CGRP, and furthermore, that the effects were directly due to the CGRP, since the CGRP antagonist reversed that result.

24. I confirm that the experiment described in Exhibit A was completed on August 26, 1999. I also confirm that on that day, I recognized that the data showed that the administration of CGRP to allergen-sensitized and challenged mice inhibited allergen-induced airway hyperresponsiveness (AHR) in the mice, as compared to in the absence of administration of CGRP. I also confirm that on that day, I recognized that the data from that experiment demonstrated that administration of CGRP to a mouse that has allergen-induced AHR inhibited allergen-induced AHR in the mouse as compared to in the absence of

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administration of CGRP. It is my opinion that anyone skilled in the relevant field reading these data would have arrived at the same conclusion on August 26, 1999.

25. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

2/23/08
Date

Arihiko Kanehiro
Arihiko Kanehiro

EXHIBIT A

Experiment 23JL99

Azd/Ari

Objective: to study the effect of CGRP on MCh-induced AHR in mouse model of asthma

Hypothesis: CGRP inhibits MCh-induced AHR in mice sensitized and exposed to OVA

Balb/c mice were sensitized to OVA (2 i.p.) on day 0 and day 14. On day 28, mice were exposed to aerosolized OVA (1%) for 20 min per day on 3 consecutive days. Control animals were not sensitized and not challenged. At 2 h prior to MCh responsiveness, animals pretreated either with i.p. injection of CGRP (200 μ l of 10^{-6} M) or i.p. injection of CGRP antagonist (100 μ l of 10^{-5} M) followed by injection of CGRP (200 μ l of 10^{-6} M). After measurement of MCh-induced AHR, the lungs were inflated with OCT:PFA and embedded in OCT for histology.

RESULTS (AHR)

Group 1: OVA ip/Neb + CGRP

	1	2	3	4
BL	0.58/68	0.73/62	0.47/72	0.63/62
SAL	0.79/39	0.91/50	0.65/48	0.76/50
1.56	0.84/34	1.01/48	0.70/31	0.84/46
3.12	0.94/29	1.16/38	0.91/28	1.14/25
6.25	1.36/24	1.70/22	1.25/21	1.46/21
12.5	1.32/23	2.14/16	1.72/12	2.35/12

Group 2: OVA ip/Neb + Antag + CGRP

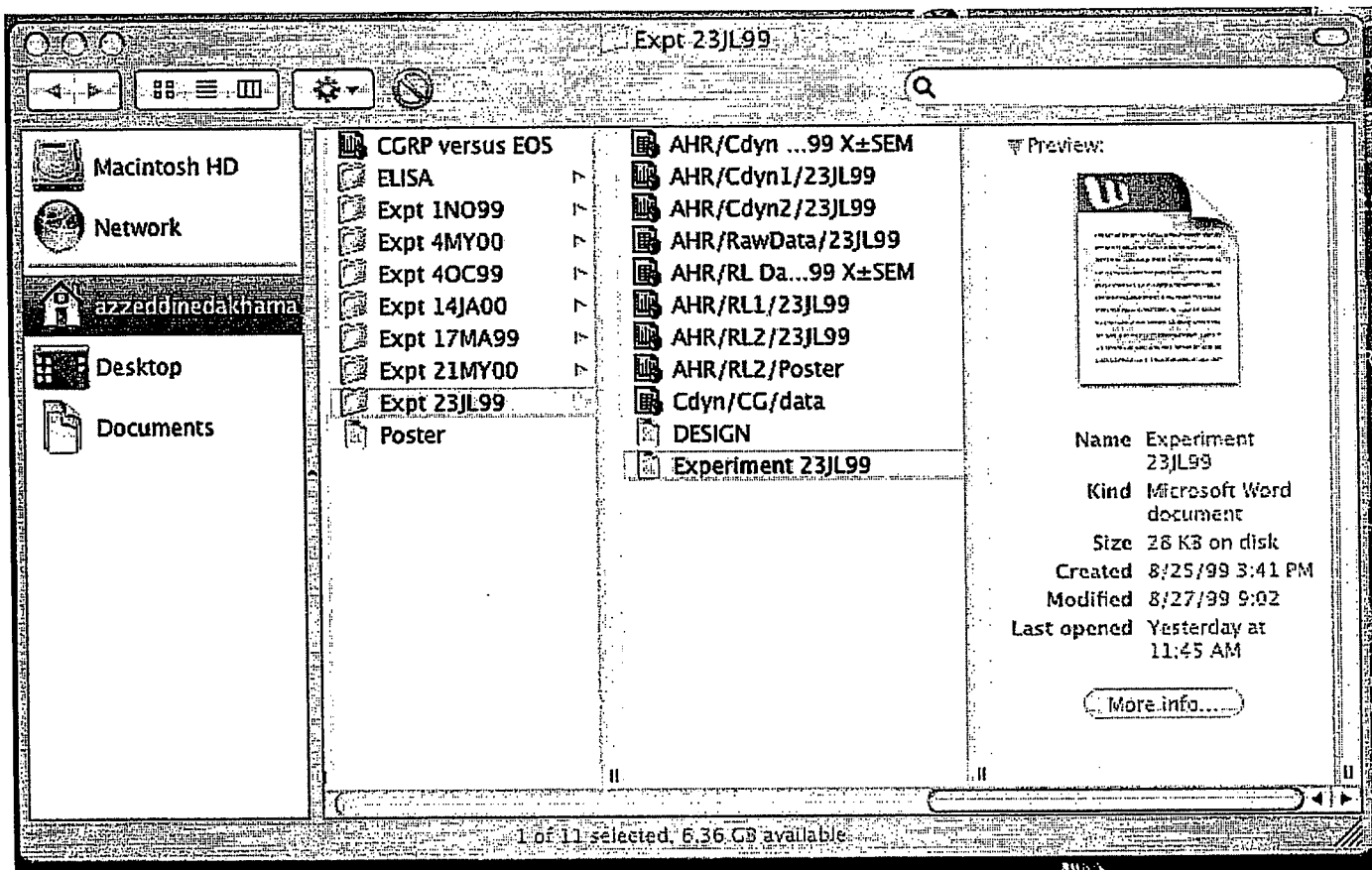
	5	6	7
BL	0.55/61	Collapsed	0.51/61
SAL	0.75/35		0.65/42
1.56	1.05/25		0.77/37
3.12	1.56/21		1.94/13
6.25	2.58/13		4.15/6
12.5	4.02/6		4.36/5

Group 3: PBS ip/Neb untreated (-CTL)

	1	2	3
BL	0.59/63	0.50/59	0.47/60
SAL	0.70/60	0.53/57	0.49/56
1.56	0.81/58	0.64/45	0.60/47
3.12	0.95/38	1.09/31	0.92/38
6.25	1.25/27	1.35/26	1.70/19
12.5	1.40/25	1.75/15	1.48/20

Group 4: OVA ip/Neb untreated (+CTL)

	1	2	3
BL	0.64/64	0.57/72	0.60/66
SAL	0.76/41	0.66/60	0.71/47
1.56	1.07/34	0.90/37	0.96/33
3.12	1.64/21	2.00/8	1.78/16
6.25	2.87/11	3.45/8	3.33/8
12.5	5.03/7	4.20/4	4.25/5



Expt 23 JUL 99

8/25 - ①

with AEP

① 1PN
+ chirp

②

③

BW

28

22.2

27

BL 0.58/68

0.73/62

0.47/72

sal 0.79/39

0.91/50

0.65/48

1.56 0.84/34

1.01/48

0.70/31

3 0.94/29

1.16/38

0.91/28

6.25 1.36/34

1.70/22

1.25/21

12.5 ~~1.17/20~~

1.32/23

2.14/16

1.72/12

$\frac{2}{28} - ②$

④

5

⑤

1PN
+ 2-MAV
+ CRRP

⑥

4

⑦

9

BW

28

26.5

33.5

30

BL 0.63/62

0.55/61

0.57/61

5.2 0.76/50

0.75/35

0.65/42

1.56 0.84/46

1.05/28

0.77/37

3 1.14/28

1.56/21

1.94/13

6.25 1.46/21

2.58/13

4.15/6

12.5 2.35/12

4.02/6

4.36/5

8/26 ①

with AZD

①

②

③

Salin Nap.

800

U.S.

U.S.

U.S.

BL 0.59/63

0.50/59

0.47/60

sal 0.70/60

0.53/57

0.48/56

1.58 0.81/58

0.64/45

0.60/47

U.S. 0.95/38

1.09/31

0.92/38

6.25 1.25/27

1.35/26

1.70/19

12.5 1.40/25

1.75/15

1.48/20

$\Delta 6$ (4)

(4) ~~00A~~ / PN (5)

(6)

(7)

BD

27

W

26

BL 0.64/04 0.57/72 0.60/66

sal 0.76/4 0.66/60 0.71/47

1.5 1.07/04 0.90/07 0.96/W

W 1.64/2 2.00/P 1.78/16

6 2.87/11 W. 45/P W. 45/8

12 5.03/7 4.20/4 4.25/5

EXHIBIT B

Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography

E. HAMELMANN, J. SCHWARZE, K. TAKEDA, A. OSHIBA, G. L. LARSEN, C. G. IRVIN,
and E. W. GELFAND

Divisions of Basic Sciences and Pulmonary Medicine, Department of Pediatrics; and Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado

To study the mechanisms and kinetics underlying the development of increased airway responsiveness (AR) after allergic sensitization, animal models have been invaluable. Using barometric whole-body plethysmography and increases in enhanced pause (Penh) as an index of airway obstruction, we measured responses to inhaled methacholine in conscious, unrestrained mice after sensitization and airway challenge with ovalbumin (OVA). Sensitized and challenged animals had significantly increased AR to aerosolized methacholine compared with control animals. AR measured as Penh was associated with increased IgE production and eosinophil lung infiltration. In a separate approach we confirmed the involvement of the lower airways in the response to aerosolized methacholine using tracheotomized mice. Increases in Penh values after methacholine challenge were also correlated with increased intrapleural pressure, measured via an esophageal tube. Lastly, mice demonstrating AR using a noninvasive technique also demonstrated increased pulmonary resistance responses to aerosolized methacholine when measured using an invasive technique the following day in the same animals. The increases in Penh values were inhibited by pretreatment of the mice with a β_2 -agonist. These data indicate that measurement of AR to inhaled methacholine by barometric whole-body plethysmography is a valid indicator of airway hyperresponsiveness after allergic sensitization in mice. The measurement of AR in unrestrained, conscious animals provides new opportunities to evaluate the mechanisms and kinetics underlying the development and maintenance of airway hyperresponsiveness and to assess various therapeutic interventions. **Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography.** *AM J RESPIR CRIT CARE MED* 1997;156:766-775.

Airway hyperresponsiveness (AHR), airway inflammation, and reversible airway obstruction are the hallmarks of bronchial asthma (1). In the development of AHR, neurogenic abnormalities (2) and airway inflammation (3), characterized by eosinophil infiltration (4), and the release of inflammatory mediators and cytokines (5) have been implicated. Animal models have been developed to investigate the pathogenetic mechanisms involved in the development of AHR, providing a means for *in vivo* manipulation and *in vitro* study of easily accessible cells and tissue (6). These models also permit the testing of different protocols and reagents for the prevention of AHR and airway inflammation, approaches that are unsuitable or impossible to perform in patients. Because of the advanced

understanding of the immune system in mice and the availability of reagents and genetically altered mice, murine models of AHR have become increasingly important in defining which cells and factors are involved (7). Several reports have described the roles that interleukin-4 (IL-4) (8), IL-5 (9, 10), and eosinophil lung infiltration (11, 12) play in the development of AHR in allergen-sensitized mice, but additional studies are needed to better define the kinetics and mechanisms underlying AHR.

To date, three different approaches have been used to measure altered airway function in mice: *in vitro* measurement of tracheal smooth muscle contractility after electrical field stimulation (13), *in vivo* measurement of lung resistance or compliance after intravenous injection of bronchoconstrictive agents such as methacholine and serotonin (14, 15), and *in vivo* measurement of peak airway opening pressure (16). Each of these methods have their limitations. The *in vitro* technique correlates well with allergic airway sensitization (13) and appears to reflect increased acetylcholine release caused by M_2 receptor dysfunction in sensitized animals (17). However, the influence of mucus production, mucosal edema, or other changes in the lower airways after allergic sensitization are not reflected in monitoring airway responsiveness with this technique. The *in vivo* techniques (14-16) perform measurements of AHR in tracheotomized and ventilated animals. The influence of anes-

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thetia and of the operating procedures on the readings is not well defined. Furthermore, intravenous challenge of the mice with bronchoconstrictive agents might not solely reflect physiologic stimulation of airway smooth muscles. Finally, this method is technically demanding and time-consuming.

In this report, we present data from studies carried out using barometric whole-body plethysmography (WBP) for the measurements of AR in unrestrained and conscious mice after sensitization and airway challenge with allergen. WBP has several potential advantages when compared with the above-mentioned techniques: it is technically not as demanding, allows measurements of AR to aerosolized stimulants, and provides a technique for repeated and long-term measurements of AR, as killing of the mice after the measurements is not required, thus allowing the evaluation of kinetics and treatment protocols of AHR. However, because of the indirect and noninvasive measurement of airway function, thorough evaluation of WBP is necessary before it can be accepted as a technique to measure AHR. The influence of upper airway responsiveness and changes in breathing pattern (respiratory rate, tidal volume) on the read-out of WBP need to be evaluated. We addressed these problems by measuring AR by WBP in tracheotomized animals, simultaneously measuring WBP and intrapleural pressure, and sequentially measuring WBP and lung resistance in the same animals. Further, we studied the effects of changes in the respiratory rate and of the response to a bronchodilator on WBP. The data shown in this study indicate that WBP in mice provides a valid assessment of AHR in allergen-sensitized mice.

METHODS

Animals

Female BALB/c mice 8 to 12 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Center for Immunology and Respiratory Medicine.

Sensitization and Airway Challenge

Groups of mice (three to four mice/group/experiment) receiving the following treatment were studied: (1) no treatment (N); (2) sensitization to OVA plus airway challenge with PBS (ip); (3) sham-sensitization with PBS plus airway challenge with OVA (Neb); (4) sensitization plus challenge with OVA (ipNeb). Mice were sensitized by intraperitoneal injection of 20 μ g OVA (Sigma, St. Louis, MO) emulsified in 2 mg aluminum hydroxide (AlumInject; Pierce Chemical, Rockford, IL) in a total volume of 100 μ l on Days 1 and 14. Mice were challenged via the airways with OVA (1% in PBS) or PBS for 20 min on Days 28, 29, and 30 by ultrasonic nebulization and assessed on Day 31 for AR. In selected mice, invasive methods to measure pulmonary resistance were employed on Day 32.

Determination of Airway Responsiveness

AR was measured in unrestrained animals by barometric plethysmography (18) using whole body plethysmography (WBP) (Figure 1) (Buxco, Troy, NY). Before taking readings, the box was calibrated with a rapid injection of 150 μ l air into the main chamber. Measured were pressure differences between the main chamber of the WBP containing the animal, and a reference chamber (box pressure signal). This box pressure signal is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. A pneumotachograph with defined resistance in the wall of the main chamber acts as a low-pass filter and allows thermal compensation (Figure 1). The time constant of the box was determined to be approximately 0.02 s.

Inspiration and expiration are recorded by establishing start-inspiration and end-inspiration as the box pressure/time curve crosses the

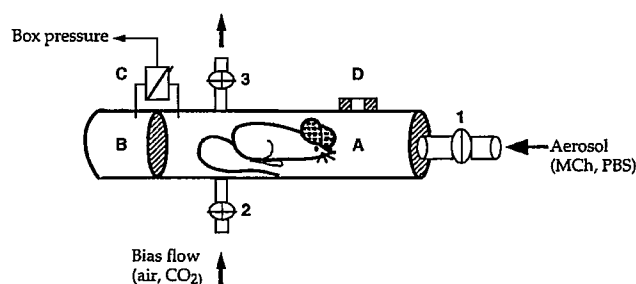


Figure 1. Schematic diagram of the whole-body plethysmograph. (A) Main chamber containing the mouse. (B) Reference chamber. (C) Pressure transducer connected to analyzer. (D) Pneumotachograph. (1) Main inlet for aerosol closed by valve. (2) Inlet for bias flow with four-way stopcock; (3) Outlet for aerosol with four-way stopcock.

zero point (see Figure 2). Start of an inspiration is determined by extrapolating from a straight line drawn from two levels of the rising inspiratory phase of the box pressure signal. Time of inspiration (T_i) is defined as the time from the start of inspiration to the end of inspiration; time of expiration (T_e) as the time from the end of inspiration to the start of the next inspiration (Figure 2). The maximum box pressure signal occurring during one breath in a negative or positive direction is defined as peak inspiratory pressure (PIP) or peak expiratory pressure (PEP), respectively (Figure 2). Recordings of every 10 breaths are extrapolated to define the respiratory rate in breaths per minute. The relaxation time (T_r) is defined as the time of pressure decay to 36% of the total expiratory pressure signal (area under the box pressure signal in expiration). This may thus serve as a correlate to the time constant (RC) of the decay of the volume signal to 36% of the peak volume in passive expiration. During bronchoconstriction, the main alteration in the signal occurs during early expiration and leads to changes in the waveform of the box pressure signal (19, 20). This

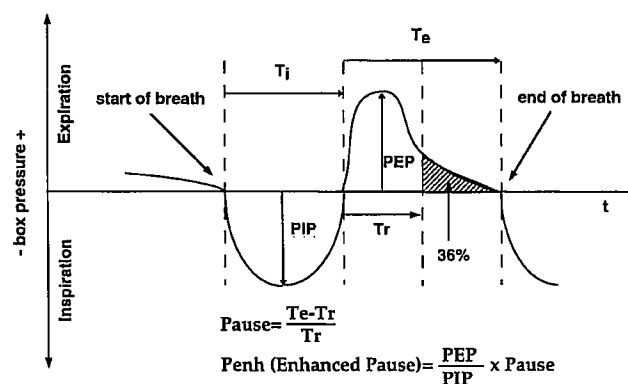


Figure 2. Computation of the parameters measured by barometric plethysmography. Schematic figure of a box pressure wave in inspiration (down) and expiration (up) explaining the computation of the parameters measured by WBP. T_i = inspiratory time (s), time from start of inspiration to end of inspiration; T_e = expiratory time (s), time from end of inspiration to start of next inspiration; PIP = peak inspiratory pressure (ml/s), maximal negative box pressure occurring in one breath; PEP = peak expiratory pressure (ml/s), maximal positive box pressure occurring in one breath; f = frequency (breaths/min), respiratory rate; T_r = relaxation time (s), time of the pressure decay to 36% of total box pressure during expiration.

change in the waveform can be quantified comparing the mean expiratory box pressure during early expiration (MP1) with the mean expiratory box pressure during late expiration (MP2) by measurement of Pause (Figure 2) where MP1 = mean expiratory box pressure 1; MP2 = mean expiratory box pressure 2; P = expiratory box pressure:

$$\begin{aligned} \text{MP1} &= \frac{0.65 P}{T_r} \\ \text{MP2} &= \frac{0.35 P}{T_e - T_r} \\ \text{Pause} &= \frac{T_e - T_r}{T_r} = \frac{0.35 P}{0.65 P} \times \frac{\text{MP1}}{\text{MP2}} - \frac{\text{MP1}}{\text{MP2}} \end{aligned}$$

During bronchoconstriction, the changes in box pressure during expiration (PEP) are more pronounced than during inspiration (PIP) (20) (see Figure 3). This is reflected by the formula for enhanced pause (Penh), a dimensionless value used in this study to empirically monitor airway function:

$$\text{Penh} = \text{Pause} \times \frac{\text{PEP}}{\text{PIP}}$$

Penh reflects changes in the waveform of the box pressure signal from both inspiration and expiration (PIP, PEP) and combines it with the timing comparison of early and late expiration (Pause). Penh is not a function of the absolute box pressure amplitude or the respiratory rate, but rather a junction of the proportion of the pressure signal from

inspiration and expiration and of the timing of expiration. An example of the box pressure waveform from a normal mouse before and after challenge with aerosolized methacholine is shown in Figure 3, demonstrating the changes in the waveform as well as in Pause and Penh after agonist inhalation.

Mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized PBS or methacholine in increasing concentrations (3 to 50 mg/ml) were nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. Airway reactivity was expressed as a fold increase for each concentration of MCh (Penh_{MCh}) compared with Penh values after PBS challenge (Penh_{PBS}).

For the quantification of the dose-response to methacholine, the linear regression of Penh on log base 2 was calculated for individual mice. The log dose corresponding to an increase in Penh of 100 or 200%, respectively, was determined, and the average log doses of the different groups were compared by analysis of variance. The data are reported as the geometric mean with the lower and upper limit of 95% confidence interval.

Studies with Mechanical Ventilation

In order to determine the influence of breathing frequency and tidal volume as well as bronchoconstriction under controlled conditions,

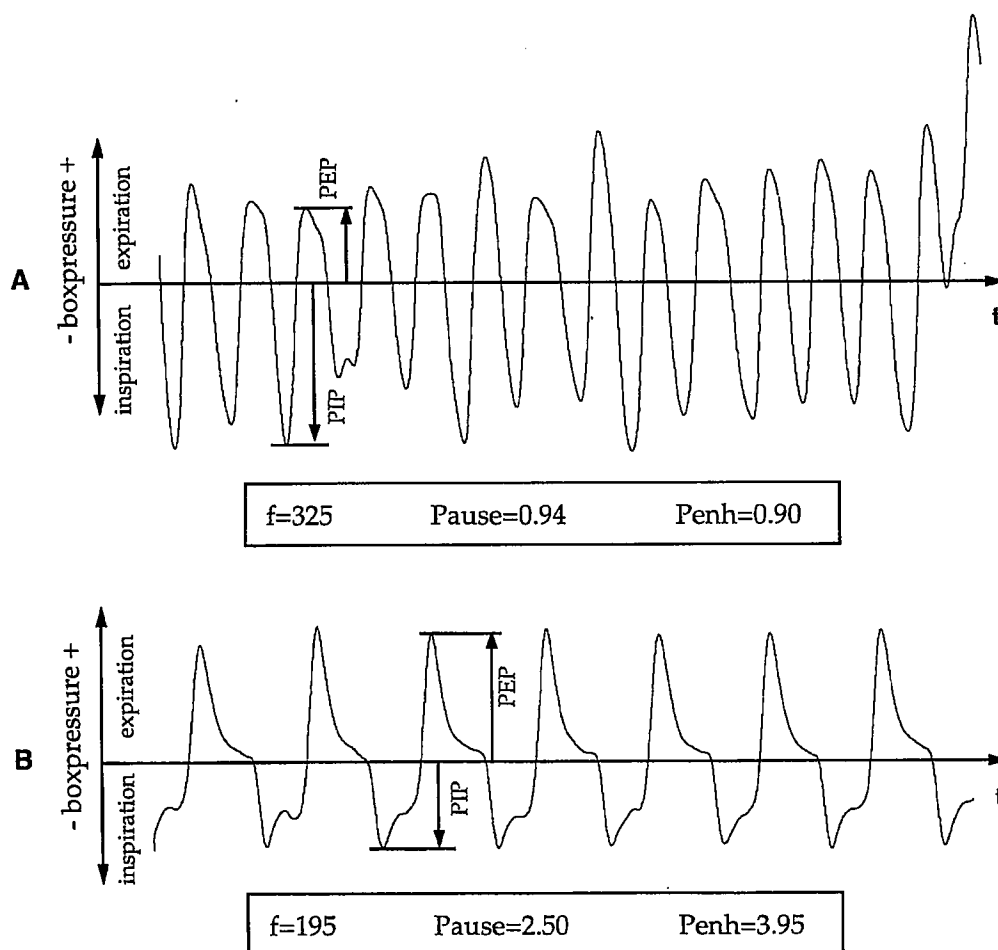


Figure 3. Changes in box pressure waveform after methacholine challenge. Waveform of the box pressure signal derived from a normal mouse after 3 min of nebulization with aerosolized PBS (A) or aerosolized methacholine (50 mg/ml in PBS) (B). *f* = respiratory rate (breaths/min); Pause, Penh (enhanced pause), PIP and PEP: see Figure 2 for description.

we performed the following studies. Mechanical ventilation was achieved by using a computer-controlled high-speed volume ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). The piston of this ventilator is connected to a linear pumping motor and a linear displacement transducer (21). By measuring the precise position of the shaft and accounting for gas compression, the volume delivered within the mouse or plethysmograph is defined and known. We then ventilated either the empty WBP or the live mice over a frequency (f) rate of 100 to 300 breaths/min and tidal volume ranges of 0.1 to 0.3 ml. We performed a tracheostomy and then connected the mice through the well of the plethysmograph to the ventilator. To investigate the effects of agonist inhalation in ventilated mice, the animals were challenged with MCh (25 mg/ml) given either intratracheally with f set to 60 breaths/min, tidal volume of 500 μ l for 30 s, or intraperitoneally after establishing PBS baselines, and Penh was measured under fixed ventilation settings (f = 300 breaths/min; tidal volume = 150 μ l).

Correlation to Respiratory Rate in Conscious Mice

To investigate if there is a relationship between Penh and respiratory rate in conscious, unrestrained, and spontaneously breathing mice, we investigated the effects of CO₂ inhalation. Normal mice were set in the main chamber of the WBP, and Penh baseline readings were taken for the first 3 min. A steady bias flow with normal air (1 L/min) was established through an additional inlet of the main chamber to allow long-term online readings. A second baseline reading of 3 min was measured after the mice were resting within the box for 30 min. Bias flow was then changed to 8% CO₂ in air (1 L/min), and respiratory rate and Penh were measured after 15 min.

Lower Airway Responsiveness

To document involvement of the lower airways in the measurements of Penh, mice were anesthetized intraperitoneally (0.3 ml 2.5% avertin in PBS) and 5-mm-long sterile plastic tubes were inserted into the tracheas and fixed by suture. PBS, MCh challenge, and measurements were performed in the spontaneously breathing mice in the WBP as described above.

Correlation to Pleural Pressure

In an attempt to directly correlate bronchoconstriction to the index derived by barometric plethysmography, intrapleural pressure changes were measured simultaneously with measurements of Penh. Saline-filled tubes were inserted into the esophagus of anesthetized mice and connected to a pressure transducer (Model MC1; Validyne, Northridge, CA). Mice were challenged with nebulized PBS and increasing concentrations of MCh as above. Changes in intraesophageal pressure (ΔP), which reflect changes in intrapleural pressure, were calculated for each MCh concentration. Changes in box pressure and respiratory frequency were measured by WBP. ΔP and Penh were recorded simultaneously and expressed as a fold increase over values after PBS challenge.

Correlation to Pulmonary Resistance

To correlate Penh with a measurement of lung resistance, *in vivo* pulmonary resistance (R_L) was measured in anesthetized, tracheostomized, and ventilated mice as previously described (14, 15). A four-way connector was attached to the tracheostomy tube, with two ports connected to the inspiratory and expiratory sides of the ventilator (Model 683; Harvard Apparatus, South Natick, MA). Ventilation was achieved with a rate of 160 breaths/min, tidal volume of 150 μ l during recording, and with a rate of 60 breaths/min, tidal volume of 500 μ l during MCh challenge. As a modification to previous work from our laboratory (15), MCh was administered as an aerosol for the period of 10 breaths for each concentration via the tracheostoma. Change in pressure, flow, and volume were recorded, and R_L was calculated from peak values after each challenge. Penh was first measured on Day 31 of the protocol and *in vivo* R_L was obtained in the same animals 1 d later.

Effects of a Beta Agonist

To study the effects of an inhaled β_2 -agonist on measurement of Penh in allergen-sensitized and challenged mice, albuterol was nebulized as an aerosol for 3 min into the main chamber followed by a 3-min read-

ing. The control group consisted of sham-treated mice aerosolized with PBS for 3 min instead of albuterol. Six minutes later, MCh was aerosolized at a 50 mg/ml concentration followed by a 6-min reading. In a different set of experiments, the effects of albuterol on repeated MCh challenge was investigated. After establishing PBS baseline values, mice were challenged with MCh (50 mg/ml) for 3 min and Penh was recorded for 6 min. The mice were divided into two groups receiving nebulization with either PBS or albuterol for 3 min followed 6 min later by a second MCh challenge (50 mg/ml) for 3 min.

Measurement of Anti-OVA Antibody and Total Ig Levels

Anti-OVA Ig serum levels were measured by ELISA as previously described (22). The antibody titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter (EU/ml). Total IgE and IgG levels were determined using the same method compared with known mouse IgE or IgG standards (PharMingen, San Diego, CA). The limits of detection were 100 pg/ml for IgE and 1 ng/ml for IgG.

Bronchoalveolar Lavage (BAL) and Lung Cell Isolation

Lungs were lavaged via a tracheal tube with Hank's balanced salt solution (HBSS, 3 \times 0.5 ml), and the cells in the lavage fluid were counted. Lung cells were isolated as previously described (22). Cells from BAL or lungs were resuspended in HBSS and counted with a hemocytometer. Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting at least 300 cells by light microscopy.

Statistical Analysis

Analysis of variance was used to determine the level of difference between all groups. Single pairs of groups were compared by Student's *t* test. Comparisons for all pairs were performed by Tukey-Kramer HSD test; *p* values for significance were set to 0.05. Values for all measurements are expressed as the mean \pm standard deviation (SD) except for values for airway reactivity (Penh, resistance, impedance), which are presented as the mean \pm standard error of the mean (SEM).

RESULTS

Noninvasive AR Increases after Methacholine Challenge in Allergen-sensitized and Challenged Mice

We established a mouse model of systemic sensitization and airway challenge with allergen, monitoring airway responsiveness using WBP in unrestrained and conscious mice. Sensitization with OVA followed by airway challenge significantly increased serum levels of anti-OVA IgE and IgG₁ and enhanced production of total IgE in BALB/c mice compared with non-sensitized control mice receiving no treatment or OVA airway challenge on two consecutive days (Table 1). In addition, all of the sensitized and challenged mice developed allergen-specific immediate cutaneous responsiveness to intradermal injections of OVA; no responses were observed in nonsensitized control mice without or with airway challenge (data not shown).

We compared the responses to inhaled MCh in the four groups of mice: untreated mice (N), sensitized and PBS-challenged mice (ip), nonsensitized and OVA-challenged mice (Neb), and sensitized and OVA-challenged mice (ipNeb). The control groups (N, Neb, ip) showed similar albeit shallow, dose-dependent increases in Penh in response to aerosolized MCh compared with the Penh values after PBS (Figure 4). In contrast, in mice that were sensitized and challenged with allergen via the airways (ipNeb), the increase in Penh in response to aerosolized MCh was significantly enhanced compared with the control mice. The MCh doses required for 100 and 200% increases in Penh were significantly reduced for sensitized and challenged mice by \sim 3.5-fold and \sim 5-fold, respectively, shifting the dose-responses leftwards compared

TABLE 1
OVA-SPECIFIC ANTIBODY AND TOTAL Ig LEVELS IN THE SERUM

Group	Sensitization	Challenge	OVA-specific (EU/ml) [†]		Total Ig (ng/ml)	
			IgE	IgG ₁	IgE	IgG
N	None	None	< 10	< 10	14.7 ± 1	243 ± 65
Neb	None	OVA	< 10	< 10	16 ± 3	235 ± 68
ipNeb	OVA	OVA	7,622 ± 736 [§]	1,029 ± 369 [§]	48.8 ± 10 [§]	265 ± 86

Definition of abbreviations: OVA = ovalbumin; N = serum titers for OVA-specific and total antibodies, which were determined by ELISA in untreated mice (n = 8); Neb = nonsensitized, OVA-challenged mice (n = 12); ipNeb = OVA-sensitized, OVA-challenged mice (n = 18). Presented are the means ± SD (OVA-specific in ELISA units/ml, total Ig in ng/ml) from three independent experiments.

* Presented are the means ± SD from the three independent experiments.

[†] ELISA units per milliliter.

[§] Significant (p < 0.01) differences compared with control group (N).

with nonsensitized control mice (Table 2). The responses peaked at 1.5 to 2 min after the challenge with aerosolized MCh, and Penh returned to prenebulization values after ~ 3 min for MCh doses ≤ 12 mg/ml and after ~ 5 to 7 min after higher doses. The Penh baseline readings after PBS were similar for all three control groups, but they were higher for sensitized and challenged animals (Figure 4). These data indicate that Penh values are increased in allergen-sensitized, airway-challenged animals. Furthermore, the response to MCh was greater in this group of mice.

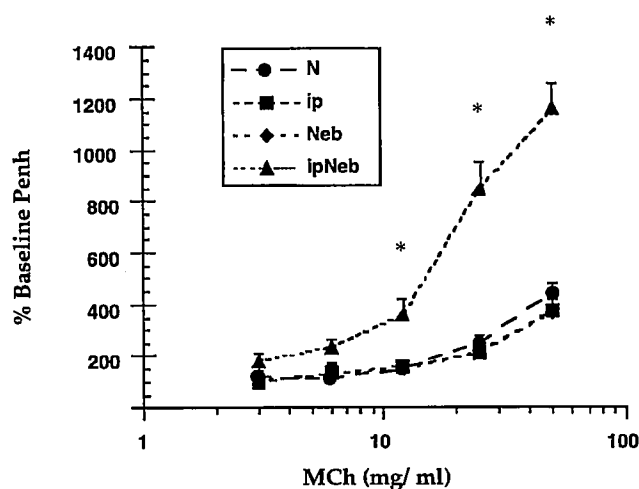


Figure 4. Penh increases in allergen-sensitized and challenged mice. Animals were sensitized with OVA/alum ip on Days 1 and 14 and challenged with OVA via the airways on Days 28, 29, and 30. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber of the WBP and were nebulized first with PBS, then with increasing doses (3 to 50 mg/ml) of methacholine for 3 min after each nebulization, followed by readings of breathing parameters for 3 min after each nebulization with PENH values determined. Compared are nontreated (N) (n = 8), nonsensitized, challenged (Neb) (n = 12), sensitized, nonchallenged (ip) (n = 12), and sensitized, challenged (ipNeb) (n = 12) mice. Expressed are the means ± SEM of the Penh values in percentages of Penh values after PBS nebulization of three independent experiments. Penh_{PBS}: N, 0.84 ± 0.03; Neb, 0.85 ± 0.03; ip, 0.81 ± 0.04; ipNeb, 1.04 ± 0.05; p < 0.05. *p < 0.01 compared with controls values.

Noninvasive AR Increases after Methacholine Challenge in Ventilated Mice

To study the impact of changes in breathing frequencies (f) and tidal volumes, we measured Penh under conditions where mice were mechanically ventilated. First, the empty WBP was connected to the ventilator and Penh was measured over the frequency range of 100 to 300 breaths/min and the tidal volume range of 100 to 300 µl. Both f and tidal volume were measured correctly by the WBP, and recorded values for tidal volume did not change under different f. Penh during mechanical ventilation changed less than 10% with various f in measurements performed in live, ventilated mice. Penh values increased nearly proportionally with increasing tidal volume in a range from 100 to 250 µl. The < 2-fold increase in Penh observed in ventilated mice (0.26 at 100 µl to 0.44 at 250 µl) was due to an increase in the Pause (0.34 to 0.54) resulting from a decrease in Tr (from 0.09 s at 100 µl to 0.07 s at 250 µl) and to a lesser extent by a decrease of T_E (from 0.12 s to 0.11 s). The decrease in Tr may be explained by the greater elastic recoil/smaller compliance of the lungs when ventilated with a higher tidal volume. These data further indicate that the volume dependency of Penh does not account for the much greater changes (> 10-fold increase versus baseline Penh) seen in sensitized challenged mice with similar changes in tidal volume (see Table 3).

Next, we measured the changes in Penh after challenge with MCh under ventilated conditions. Normal, anesthetized mice were ventilated and challenged with MCh (25 mg/ml) either intratracheally or intraperitoneally. Under both conditions, the mice developed a more than 200% increase in Penh

TABLE 2
DOSE-RESPONSE OF PENH TO MCh

Group	Sensitization	Challenge	MCh Dose (mg/ml) Required for an Increase in Penh of			
			100%	(LL; UL)	200%	(LL; UL)
N	None	None	17.7	10.5; 16	32	21; 48.5
Neb	None	OVA	18.7	11.5; 30.4	42	32; 105
ipNeb	OVA	OVA	5.2 [†]	4.4; 6.1	7 [†]	5.6; 8.3

Definition of abbreviations: Penh = plethysmography and increases in enhanced pause; MCh = methacholine; N = dose-response in Penh after challenge with aerosolized MCh in untreated mice (n = 8). For other definitions, see Table 1.

* Reported is the geometric mean and the lower (LL) and upper (UL) level of the 95% confidence interval in mg/ml of MCh required for an increase of Penh to 100 or 200% compared with PBS-baseline values.

[†] p < 0.01 versus control groups (N and Neb).

TABLE 3
EFFECTS OF METHACHOLINE OR AVERTIN ON
BREATHING PATTERNS AND PENH*

Group	Anesthesia	Challenge (nebulized)	Respiratory Frequency	Tidal Volume	Penh
ipN	None	PBS	320 ± 12 [†]	0.25 ± 0.03 [†]	1.01 ± 0.06 [†]
ipN	None	MCh 50	165 ± 24	0.4 ± 0.04	11.4 ± 2.6
ipN	Avertin	PBS	189 ± 21	0.35 ± 0.04	1.04 ± 0.2

* Correlation between breathing patterns and Penh values. Expressed are the mean ± SD values of respiratory frequency (in breath/min), tidal volume (in μ l), and Penh after PBS and MCh (50 mg/ml) challenge for conscious (n = 8) and anesthetized (avertin, n = 8), sensitized, and challenged mice.

[†] p < 0.05 versus two other groups.

[‡] p < 0.05 versus MCh challenge.

values after MCh of PBS-baseline values. This suggests that the increase in Penh cannot be accounted for by changes in f or tidal volume after challenge with MCh as these variables are controlled in mechanically ventilated mice.

Noninvasive AR Does not Correlate with Breathing Patterns and Respiratory Rate

MCh challenge of mice induces increases in Penh and decreases in respiratory rate. To study the effects of changes in breathing patterns on Penh in spontaneously breathing mice and to investigate if the observed slowing of the respiratory rate itself causes an increase in Penh, sensitized and challenged mice were anesthetized intraperitoneally with an injection of avertin (2.5% in PBS) and compared with conscious animals. In anesthetized animals, frequency was decreased to ~60% compared with that in conscious mice. However, changes in these breathing patterns were not accompanied by increases in Penh

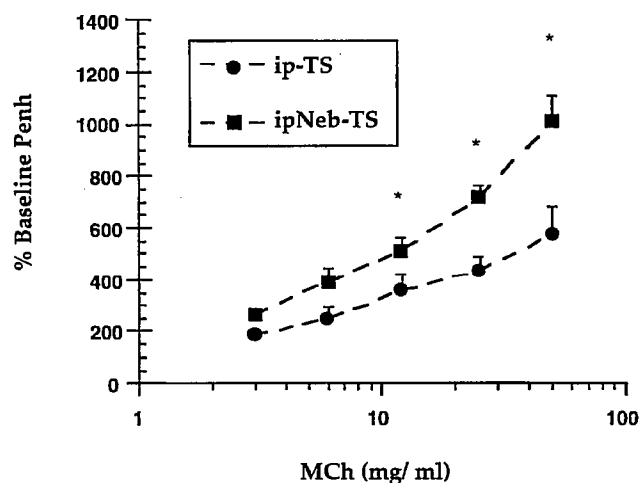


Figure 5. Penh increases after methacholine challenge of the lower airways. Mice were sensitized and challenged as described in Figure 1. Mice were tracheostomized, and airway responsiveness was measured as described in Figure 1. Compared are sensitized but not challenged (ip-TS) (n = 12) and sensitized and challenged tracheostomized mice (ipNeb-TS) (n = 12). Expressed are the means ± SEM of the Penh values in percentages of Penh values after PBS nebulization from three independent experiments. Penh_{PBS}: ip-TS, 0.94 ± 0.07; ipN-TS, 1.2 ± 0.15. *p < 0.01 compared with controls values.

(Table 3). MCh challenge (50 mg/ml) of conscious mice resulted in changes in breathing patterns similar to those observed in anesthetized animals, but they were followed by significant increases in Penh. Challenge of anesthetized mice with MCh resulted in a dose-response curve similar to those seen in the conscious animals (Figures 4 and 5), although the respiratory rate of each concentration of MCh challenge in the anesthetized animals was less than that in conscious animals (data not shown).

To further study the influence of the respiratory rate in spontaneously breathing mice receiving Penh, CO₂ was used as a respiratory stimulant. After 30 min resting in the box with a steady bias flow of normal air, mice showed a significant decrease in the average respiratory rate of 25% with virtually no changes in Penh readings (Table 4). Changing the bias flow from normal air to air containing 8% CO₂ induced a significant increase in respiratory rate of 46% accompanied with a nonsignificant drop of Penh by 10% (p = 0.2). These data indicate that decreases or increases in the respiratory rate are not necessarily accompanied with changes in Penh but rather are independently regulated.

Noninvasive AR Increases after Methacholine Challenge of the Lower Airways

To address the possibility that increases in Penh are simply due to reactions of the upper airways, e.g., swelling of the nasal mucosa or increased glandular activity, we bypassed the upper airways by performing a tracheostomy (TS) in mice before challenge with aerosolized MCh. As indicated in Figure 5, challenge of these mice with nebulized MCh resulted in dose-dependent increases in Penh. In sensitized and challenged mice (ipNeb-TS), increases in Penh values were significantly enhanced. Baseline Penh values were similarly higher in ipNeb-TS than in ip-TS animals (Figure 5). The higher Penh baseline values compared with those in nontracheostomized mice is most likely due to the fixed resistance of the tracheostomy. The magnitude of the dose-response in sensitized, challenged animals after challenge with higher doses of MCh (25 and 50 mg/ml) was very similar in tracheostomized and nontracheostomized mice (Figures 4 and 5). The MCh dose required for a 100% increase in Penh was significantly decreased and shifted to the left from 4.4 (3.3; 5.9) in ip-TS to 2.9 (2.3; 3.7) in ipNeb-TS (~1.5-fold, p < 0.05), and from 16 (8; 24) in ip-TS to 4.6 (2.9; 7.8) in ipNeb-TS (~3.5 fold, p < 0.01) for a 200% increase. The smaller magnitude of the shift in that dose-response to MCh in tracheostomized compared with nontracheostomized mice might be explained by the fact that the availability of MCh is higher in tracheostomized mice because the upper airways are by-passed, which may result in higher responses in nonsensitized mice. Secondly, the data suggest that at least

TABLE 4
EFFECT OF CO₂ ON RESPIRATORY RATE AND PENH*

Condition	Time in Box (min)	Air (bias flow)	Breathing Frequency	Penh
1	3	Air	306 ± 13	1.06 ± 0.17
2	30	Air	243 ± 15 [†]	1.07 ± 0.15
3	45	Air/8% CO ₂	356 ± 13 [‡]	0.95 ± 0.2

* Correlation between respiratory frequency and Penh values. Expressed are the mean ± SD values of respiratory frequency (in breaths per min) and Penh for mice (n = 4) 3 min (Condition 1) and 30 min (Condition 2) after start of online measurements and after additional 10 min of bias flow with 8% CO₂ in air (Condition 3).

[†] p < 0.01 versus condition 1.

[‡] p < 0.01 versus Condition 2.

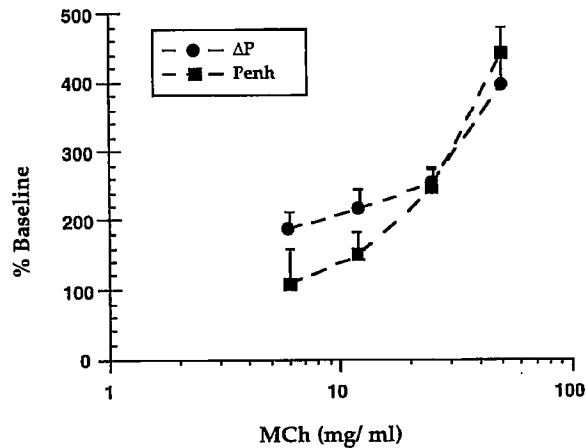


Figure 6. Increases in Penh and intrapleural pressure after methacholine airway challenge. Measurements of airway responsiveness in the WBP were performed as described in Figure 1. A saline-filled esophageal tube was connected to a second pressure transducer, and intraesophageal pressure was recorded simultaneously with PENH after each challenge with MCh. Expressed are the means \pm SEM of Penh and ΔP (intraesophageal pressure differences) as percentages of baseline values after PBS nebulization from two independent experiments ($n = 8$).

part of the increased responsiveness measured by Penh may be related to altered responsiveness of the upper airways.

Noninvasive AR Correlates with Intrapleural Pressure

To directly correlate Penh values with changes in pleural pressure occurring in the lower airways after MCh challenge, a saline-filled esophageal tube was placed in the mice to reflect changes in intrapleural pressure. Simultaneously, Penh was measured using the WBP. Mice were then challenged with aerosolized PBS or increasing doses of MCh. As shown in Figure 6, nebulized MCh induced increases in Penh similar to those in intrapleural pressure (ΔP) compared with values after PBS exposure. Moreover, the increases in Penh correlated with increases in ΔP (Figure 7B). These data demonstrate that Penh values correlate with increases in intrapleural pressure differences in the lower airways after MCh challenge.

Noninvasive AR Correlated with *in vivo* Pulmonary Resistance

To determine if increases in Penh values correlate with increased *in vivo* pulmonary resistance, we monitored Penh and pulmonary resistance in the same animals on 2 consecutive days. Pulmonary resistance was measured in intubated and ventilated mice, administering aerosolized MCh via the tracheostomy. Data were calculated from the peak values after each MCh challenge and expressed as the increase compared with measurements after PBS nebulization. Aerosolized MCh increased pulmonary resistance in a dose-dependent manner; sensitized, OVA-challenged animals (ipNeb) showed significantly higher pulmonary resistance than did nonsensitized, OVA-challenged control animals (Neb) (Figure 8B). Increases in pulmonary resistance paralleled increases in Penh values monitored by WBP in the same animals the day previously (Figure 8A). A comparison of the responses of R_L and Penh for individual mice in the same experiment (Figure 7A) indicates the

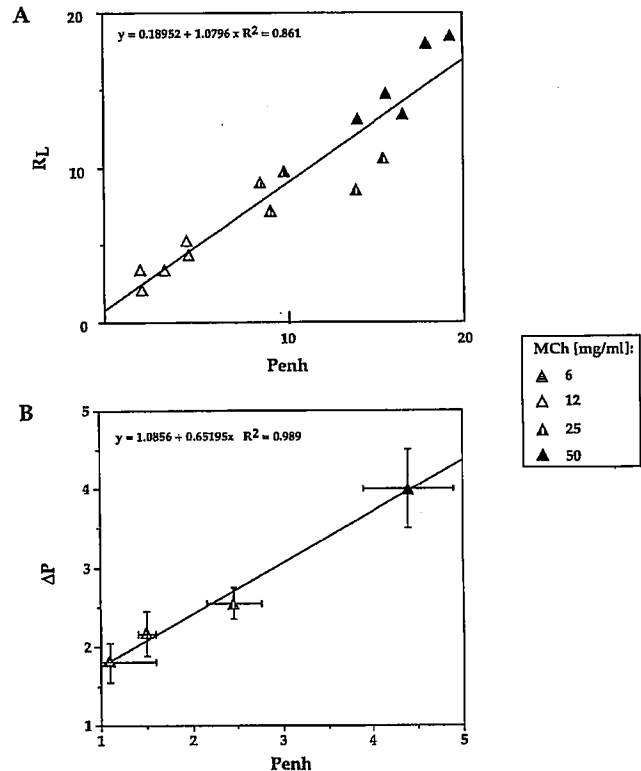


Figure 7. Penh correlates with pulmonary resistance and intrapleural pressure. Mice were sensitized and measurements of airway responsiveness were performed as described in Figure 4. Compared are the responses to aerosolized MCh measuring Penh and airway resistance (R_L) on 2 consecutive days in the same individual mice (A), or measuring Penh and ΔP simultaneously as described in Figure 3 (B). Shown are the results from one of two similar experiments (A) and the mean \pm SEM of the results from two independent experiments (B), respectively.

strong correlation between Penh and increased pulmonary resistance in sensitized and challenged mice.

Noninvasive AR Increases Are Inhibited by Albuterol

To assess the effect of β_2 -agonist on Penh, albuterol was administered by nebulization to allergen-sensitized and challenged mice after obtaining a PBS baseline. Aerosolization of albuterol did not change baseline Penh values after PBS (data not shown). MCh was then aerosolized at 50 mg/ml, and Penh was recorded for 6 min after each nebulization. Sensitized and allergen-challenged animals receiving sham-treatment with aerosolized PBS instead of the β_2 -agonist showed a significant increase in Penh values after MCh challenge (Figure 9). Pretreatment with aerosolized albuterol significantly reduced the increases in Penh values after MCh challenge. Subsequent albuterol treatment of mice that showed a more than 10-fold increase in Penh after a first MCh (50 mg/ml) challenge resulted in significantly reduced Penh values after a second MCh challenge ($650 \pm 120\%$ of PBS baseline), whereas mice receiving PBS sham-treatment instead of albuterol after a first MCh challenge showed consistently higher Penh readings after repeated MCh challenge ($1,250 \pm 150\%$ of PBS baseline). These

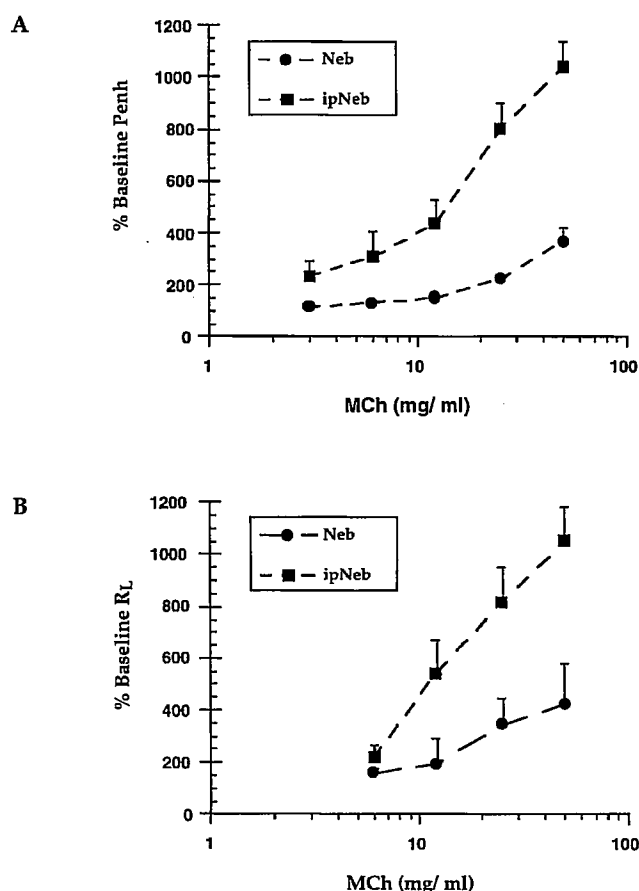


Figure 8. Increases in Penh and pulmonary resistance after methacholine airway challenge. Mice were sensitized and measurements of airway responsiveness were performed as described in Figure 1. Using the same mice, pulmonary resistance was measured in anesthetized, tracheostomized, and ventilated animals the following day. Aerosolized PBS and MCh were administered via the tracheostomy. Pulmonary resistance was calculated as $R_L = \Delta P$ (difference in tracheal pressure)/ ΔV (flow change) from peak values after each challenge. Compared are nonsensitized, challenged (Neb) ($n = 12$) and sensitized, challenged (ipNeb) ($n = 12$) mice. Expressed are the means \pm SEM of Penh (A) and of R_L (B) as percentages of baseline values after PBS nebulization. $Penh_{PBS}$: Neb, 0.86 ± 0.03 ; ipNeb, 1.09 ± 0.08 from three independent experiments * $p < 0.01$ compared with control (N).

data indicate that increases in Penh values in response to MCh are at least partially preventable after pretreatment with the bronchodilator.

Increases in Penh Are Associated with Increased Eosinophils in Lung Tissue

To correlate AR with airway inflammation, total leukocyte counts and differential counts for BAL fluid cells and isolated lung cells of individual mice were compared in the different groups. Sensitization and challenge resulted in a significant increase in eosinophils in BAL fluid ($38 \pm 4\%$) and in lung cells ($13 \pm 2\%$) compared with naive animals ($1.2 \pm 0.4\%$ in BAL and $1.1 \pm 0.5\%$ in lung cells). The increase in total numbers of eosinophils of 12-fold in lung cells and 70-fold in BAL fluid

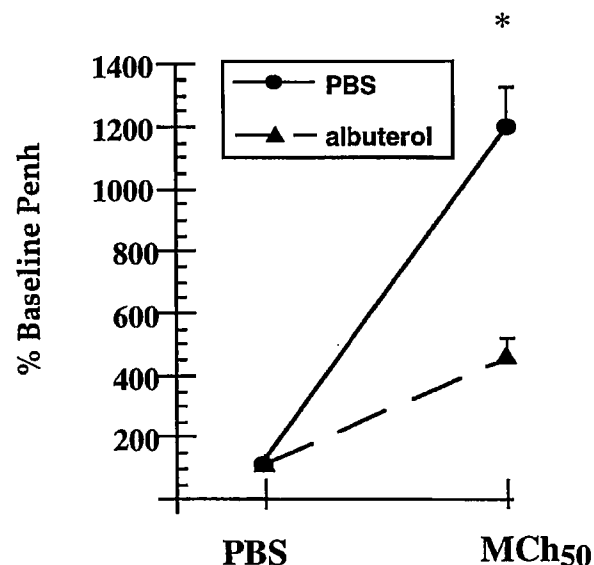


Figure 9. Penh increases are inhibited by nebulized albuterol. Mice were sensitized, and measurements of airway responsiveness were performed as described in Figure 1. After obtaining baseline Penh values after aerosolization of PBS, mice were treated with aerosolized albuterol or PBS for 3 min. After 6 min, mice were challenged with aerosolized MCh at 25 and 50 mg/ml for 3 min each, and Penh was recorded for 3 and 6 min after each nebulization. Compared are sensitized, challenged mice with albuterol ($n = 4$) or PBS ($n = 4$) treatment. Expressed are the means \pm SEM of Penh values in percentages of Penh values after PBS nebulization. $Penh_{PBS}$, 1.06 ± 0.03 ; albuterol, 1.09 ± 0.08 . * $p < 0.01$ compared with PBS sham-treated mice.

was associated with a ~ 4 -fold increase in Penh after similar MCh challenge compared with the control animals (Table 5).

DISCUSSION

In this report, we characterize a method to measure *in vivo* airway responsiveness in conscious, spontaneously breathing mice. We used a barometric whole-body plethysmograph (WBP) that measures pressure differences between a main chamber containing the animal and a reference chamber. This box pressure signal potentially detects a number of different parameters (23). Among these are heat and humidity changes that occur in the inspired and expired air. However, as the respiratory rate of the animal is 300 breaths/min (or ~ 5 Hz) and stays at ~ 120 breaths/min (or ~ 2 Hz) in bronchoconstricted animals, the

TABLE 5
INCREASES IN PENH CORRELATE WITH EOSINOPHIL LUNG INFILTRATION*

Group	Sensitization (ip)	Challenge (nebulized)	Eosinophils In		Penh After	
			BAL ($\times 10^3$)	Lungs ($\times 10^6$)	MCh 25	MCh 50
N	None	None	2.4 ± 1.3	0.7 ± 0.2	1.4 ± 0.3	2.8 ± 0.2
Neb	None	OVA	2.2 ± 1.1	0.7 ± 0.3	1.5 ± 0.2	2.9 ± 0.4
ipNeb	OVA	OVA	$173 \pm 65^\dagger$	$8.6 \pm 2.2^\dagger$	$5.3 \pm 0.9^\dagger$	$9.06 \pm 1^\dagger$

* Correlation between eosinophil numbers in the BAL fluid and the lung tissue and Penh values after allergic sensitization. Compared are nontreated (N), nonsensitized, OVA-challenged (Neb), and sensitized, challenged (ipNeb) mice. Presented are the means \pm SEM.

† Significant ($p < 0.05$) differences compared with control groups (N and Neb).

volume/flow changes are, in all likelihood, isothermal. The remaining contributors to the changes indicated by the changes of the box pressure signal are alterations in respiratory rate, tidal volume, or compression artifacts (24) (phase lags between nasal and thoracic flow). The avertin (Table 3) and CO₂ (Table 4) experiments suggest that changes in the respiratory rate are not causing similar changes in Penh as observed after agonist inhalation. Independence of Penh measurements from breathing frequency was determined in studies in mechanically ventilated mice where Penh is largely independent of frequency under ventilation with constant volumes. As some increase in Penh occurs with increasing tidal volume in mechanically ventilated mice, yet these changes cannot account for the more than 10-fold increase in Penh values observed after MCh challenge in allergen-sensitized and challenged mice. Taken together, these data suggest that this technique can be used to assess airway responsiveness in mice in a noninvasive fashion.

Bronchoconstriction is known to alter breathing patterns, and indeed changes in Pause and Penh are really due to alterations in the timing of breathing as well as a prolongation of the expiratory time. Airway constriction is further known to lead to an increase in the thoracic flow that is not synchronized with the nasal flow (25), thus resulting in an increase in the box pressure signal. The increase in the time lag between the nasal and the thoracic flow is proportional to total airway resistance and can be used to measure AR by barometric plethysmography (26–28). Penh is considered an empiric parameter that reflects changes in the waveform of the measured box pressure signal that are a consequence of bronchoconstriction. The data in this report show a close correlation between changes in indices derived from the box pressure signal (Penh) and changes in intrapleural pressure or lung resistance (R_L) to aerosolized MCh. Therefore, we conclude that under these conditions, this measurement (Penh) appears to be a valid indicator of bronchoconstriction in mice.

Several investigators have used barometric WBP to measure AHR in guinea pigs and rats (18, 26, 27). The use of this technique in mice enabled us to establish dose-response curves to an aerosolized bronchoconstrictive agent and to differentiate between normal levels of AR in control animals and hyperresponsiveness in allergen-sensitized and challenged mice. Sensitization without allergen challenge or airway challenge of nonsensitized mice was without effect on the Penh values when compared with nontreated animals. The response to MCh in sensitized and challenged animals was both shifted to the left (Table 2) and amplified (elevation of the maximal response, Figure 4) compared with that in control animals. This resembles *in vivo* AHR in patients suffering from bronchial asthma (29) and measurements of AHR with invasive methods in other animal models (30). The most likely mechanism underlying the increases in Penh is bronchoconstriction, mediated through the muscarinic receptors on smooth muscles of the airways. This is supported by the rapid but transient responses to aerosolized MCh. Further evidence for airway obstruction as the major mechanism underlying the increases in Penh was obtained using a β_2 -agonist: pretreatment of sensitized and allergen challenged animals with aerosolized albuterol significantly reduced the increases in Penh after MCh challenge. Albuterol treatment of mice that had already responded with high increases in Penh values after a first dose of MCh prevented similar responses after a second challenge with the same concentration of MCh. Importantly, changes in the respiratory rate in anesthetized mice or after CO₂-stimulation were not accompanied by changes in the Penh values, suggesting that Penh does not correlate simply with changes in breathing patterns.

One problem of measuring AHR with barometric WBP is the uncertainty of the site of obstruction (31) and the absolute value of airway resistance. We studied the effects of lower airway challenge on the development of AHR in the WBP. Lower airway challenge with MCh in tracheostomized mice resulted in a significant increase in airway responses and a shift to the left of the dose-response in allergen-sensitized and challenged animals compared with control mice. The somewhat smaller magnitude in the shift of the MCh dose-response when the upper airways are by-passed by the tracheostomy might suggest that at least a small part of the increased responsiveness as measured with Penh is related to altered upper airway responsiveness. Direct correlation between Penh and changes in lower AR was achieved in parallel measurements of Penh and intrapleural pressure after aerosolized MCh challenge. In order to correlate Penh values with pulmonary resistance, we compared the responses measured by WBP with measurements of pulmonary resistance in the same animals, obtained 1 d later. The responses monitored in the two systems were virtually identical, with comparable increases and a similar left-shift of the dose-response curve over baseline values. These data indicate that Penh correlates well with measurements of pulmonary resistance, and that WBP provides a valid measurement of AHR in allergen sensitized and challenged mice.

A number of studies have associated changes in AHR with increases in allergen-specific IgE (32, 23) and eosinophil airway infiltration (34–36). In our model of allergic sensitization, increases in specific IgE were observed. Further, after allergen challenge, increased numbers of eosinophils were detected in the BAL fluid and in isolated lung cells. Increases in Penh values were associated with production of antigen-specific IgE and the development of an eosinophil infiltration in the lungs after allergen challenge of sensitized mice. These findings confirm the association between AHR, measured by barometric WBP, and eosinophilic inflammation.

In summary, this report describes a method to monitor AHR to aerosolized MCh challenge in conscious, spontaneously breathing mice after allergen-sensitization and challenge. We have shown that changes in the box pressure signal (or the empirically derived parameter of Penh) track the changes in the respiratory system caused by bronchoconstriction. Because barometric WBP is a noninvasive technique the animals do not need to be killed once the measurements are finished, and several measurements on the same animals can be performed, allowing longitudinal studies and investigation of treatment protocols. AHR can be monitored over an extended period of time to mimic chronic allergen exposure, and the kinetics of restoration and secondary responses to allergen can be studied. In addition, this technique is potentially attractive in studying animals infected with various pathogens. Measurements of increased AHR obtained in the WBP correlated with increases in IgE serum levels, eosinophil lung infiltration, and increased lung resistance. We conclude that WBP provides a promising technique to investigate the mechanism and the kinetics underlying the development of AHR and will support the study of new approaches in the prevention of AHR.

Acknowledgment: The writers thank Dr. Peter Sly, Western University of Australia, Perth, Australia, for his help with the computer-controlled ventilation studies.

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EXHIBIT C

Development of Eosinophilic Airway Inflammation and Airway Hyperresponsiveness in Mast Cell-deficient Mice

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Summary

Mast cells are the main effector cells of immediate hypersensitivity and anaphylaxis. Their role in the development of allergen-induced airway hyperresponsiveness (AHR) is controversial and based on indirect evidence. To address these issues, mast cell-deficient mice (*W/W^u*) and their congenic littermates were sensitized to ovalbumin (OVA) by intraperitoneal injection and subsequently challenged with OVA via the airways. Comparison of OVA-specific immunoglobulin E (IgE) levels in the serum and numbers of eosinophils in bronchoalveolar lavage fluid or lung digests showed no differences between the two groups of mice. Further, measurements of airway resistance and dynamic compliance at baseline and after inhalation of methacholine were similar. These data indicate that mast cells or IgE-mast cell activation is not required for the development of eosinophilic inflammation and AHR in mice sensitized to allergen via the intraperitoneal route and challenged via the airways.

Mast cells play a central role in immediate allergic reactions (1) and in the early phase of the asthmatic response, but their role in the late phase response or sustained airway hyperresponsiveness is not clearly defined. Arming and activation of mast cells is through the binding of IgE to high affinity IgE receptors (FcεR1) on the cell surface (2, 3). After antigen cross-linking, the cells discharge a group of mediators including histamine and leukotrienes (4, 5), which trigger immediate responses. The fact that mast cells may also synthesize and secrete several cytokines on activation, including IL-4 and TNF-α, indicates their potential role in the sustained airway abnormalities. It is largely based on circumstantial evidence that mast cells are implicated in asthma pathogenesis. Because many other cell types express high or low affinity receptors for IgE and can release biologically active mediators on activation (6), a number of other cell types may be important in the IgE-dependent responses in the airways.

Mast cell-deficient mice can be used to directly assess the role of mast cells in allergen-driven airway hyperresponsiveness (AHR) (7). However, there have been limited investigations of these mice in terms of airway inflammation and the development of AHR. In the present study, we assessed the physiological response of the airways after sensitization and challenge to OVA in *W/W^u*, genetically mast cell-deficient mice to investigate more directly the role of the mast cell.

Materials and Methods

Animals. Female mast cell-deficient ([WB/Rej-kit^W/+ × C57BL6J-kit^W-v/+]F1 - [*W/W^u*]mice) (*W/W^u*) and congenic WBB6F1 normal mice (+/+) from 8 to 12 wk of age were obtained from Jackson Labs. (Bar Harbor, ME). The animals were maintained on an OVA-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Sensitization and Airway Challenge. Groups of mice (6–10 mice/group/experiment) receiving the following treatment were studied: (a) airway challenge to nebulized OVA alone (N); (b) sensitization to OVA with alum plus aerosolized airway challenge with nebulized OVA (ipN). Mice were immunized by intraperitoneal injection of 20 μg of OVA (Grade V; Sigma Chemical Co., St. Louis, MO) emulsified in 2.25 mg alum (AlumImject; Pierce, Rockford, IL) in a total volume of 100 μl on days 1 and 14. Mice were challenged via the airways with OVA (1% in saline) for 20 min on days 28, 29, and 30 by ultrasonic nebulization, and assessed 48 h after the last OVA exposure for AHR.

Determination of Airway Responsiveness. Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine (MCh) via the airways. Anesthetized, tracheostomized mice were mechanically ventilated and lung function was assessed using methods similar to those described by Martin et al. (8). A four-way connector was attached to the tracheostomy tube, with two ports connected to the inspiratory and expiratory sides of a ventilator (model 683; Harvard Apparatus, South Natick, MA). Ventilation was achieved at 160 breaths/min

and a tidal volume of 0.15 ml with a positive end-expiratory pressure of 2–4 cm H₂O.

The Plexiglas chamber containing the mouse was continuous with a 1.0-liter glass bottle filled with copper gauze to stabilize the volume signal for thermal drift. Transpulmonary pressure was detected by a pressure transducer with one side connected to the fourth port of the four-way connector and the other side connected to a second port on the plethysmograph. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer and then referenced to a second copper gauze-filled 1.0-liter glass bottle. Flow was measured by digital differentiation of the volume signal. Lung resistance (R_L) and dynamic compliance (C_{dyn}) were continuously computed (Labview, National Instruments, TX) by fitting flow, volume, and pressure to an equation of motion.

Aerosolized agents were administered for 10 s with a tidal volume of 0.5 ml (9). From 20 s up to 3 min after each aerosol challenge, the data of R_L and C_{dyn} were continuously collected. Maximum values of R_L and minimum values of C_{dyn} were taken to express changes in murine airway function.

Bronchoalveolar Lavage and Lung Cell Isolation. Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS (1 × 1 ml 37°C). The volume of collected bronchoalveolar lavage (BAL) fluid was measured in each sample and numbers of leukocytes were counted (Coulter Counter; Coulter Corporation, Hialeah, FL). Cells in lung tissue were isolated and counted as previously described (10).

Histologic and Immunohistochemistry Studies. After perfusion via the right ventricle, lungs were inflated through the trachea with 2 ml air and then fixed in 10% formalin by immersion. Blocks of the left lung tissue were cut from around the main bronchus and embedded in paraffin blocks, 5-μm tissue sections were affixed to microscope slides and deparaffinized. The slides were then stained with Astra Blue/Vital New Red and mast cells and eosinophils were examined under light microscopy (11).

Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using a rabbit anti-mouse MBP (provided by Dr. G. Gleich and Dr. J. Lee, Mayo Clinic, Rochester, MN and Scottsdale, AZ, respectively) (12). Numbers of eosinophils in the submucosal tissue around central airways were analyzed using the

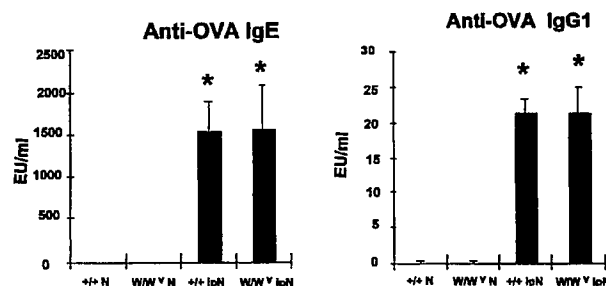


Figure 1. OVA-specific antibody in the serum. Serum titers for OVA-specific antibodies in +/+ and W/W mice were determined after sensitization and challenge ($n = 7$) compared with mice receiving challenge alone ($n = 6$). The results for each of the groups are expressed as means \pm SEM. *Significant differences ($P < 0.05$) between the groups (N versus IpN). EU, ELISA units; N, challenge (nebulization alone); ipN, sensitization and challenge.

IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh counting four different sections per animal (12).

Measurement of Anti-OVA Antibody. Serum levels of anti-OVA IgG1 and IgE were measured by ELISA as previously described (10).

Statistical Analysis. All results are expressed as the mean and SEM. Analysis of variance was used to determine the levels of difference between all groups. Pairs of groups were compared by unpaired two-tailed Student's t test. ANOVA was used to compare percent changes of R_L and C_{dyn} between different strains with the same treatment. The p values for significance were set to 0.05.

Results

Antibody Responses to OVA Sensitization and Challenge. As shown in Fig. 1, serum levels of anti-OVA IgE and IgG1 were comparable in the mast cell-deficient mice and

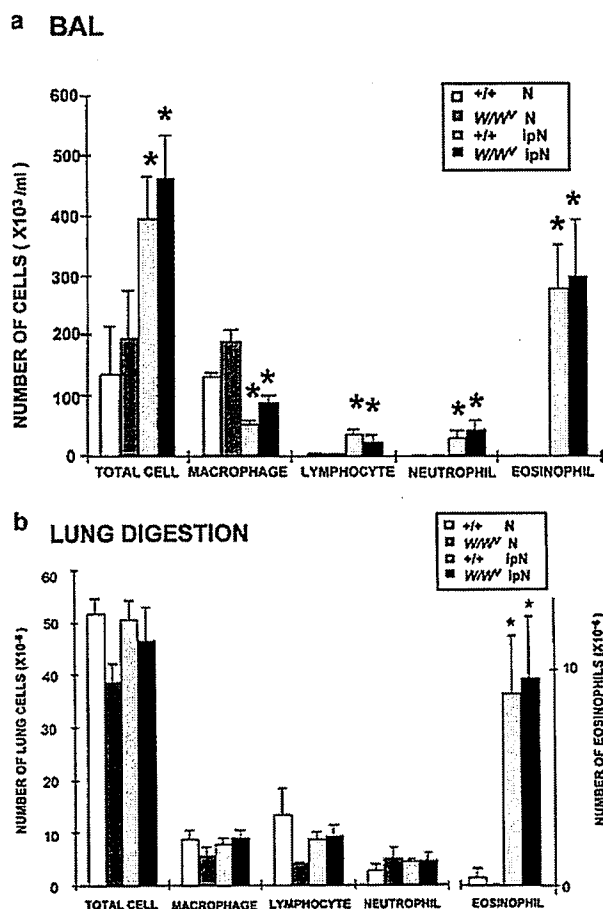


Figure 2. (a) Cellular composition of BAL fluid. Mice were sensitized and challenged as described in Materials and Methods. BAL fluid was obtained from the same groups described in the legend to Fig. 1. The results for each group are expressed as means \pm SEM. *Significant differences ($P < 0.05$) between the groups (N versus IpN). (b) Cellular composition of isolated lung cells. Lung cells were prepared from animals sensitized and challenged as described in the legend to Fig. 1. The results for each group are expressed as means \pm SEM ($n = 4$ /group). *Significant differences ($P < 0.05$) between the groups (N versus IpN).

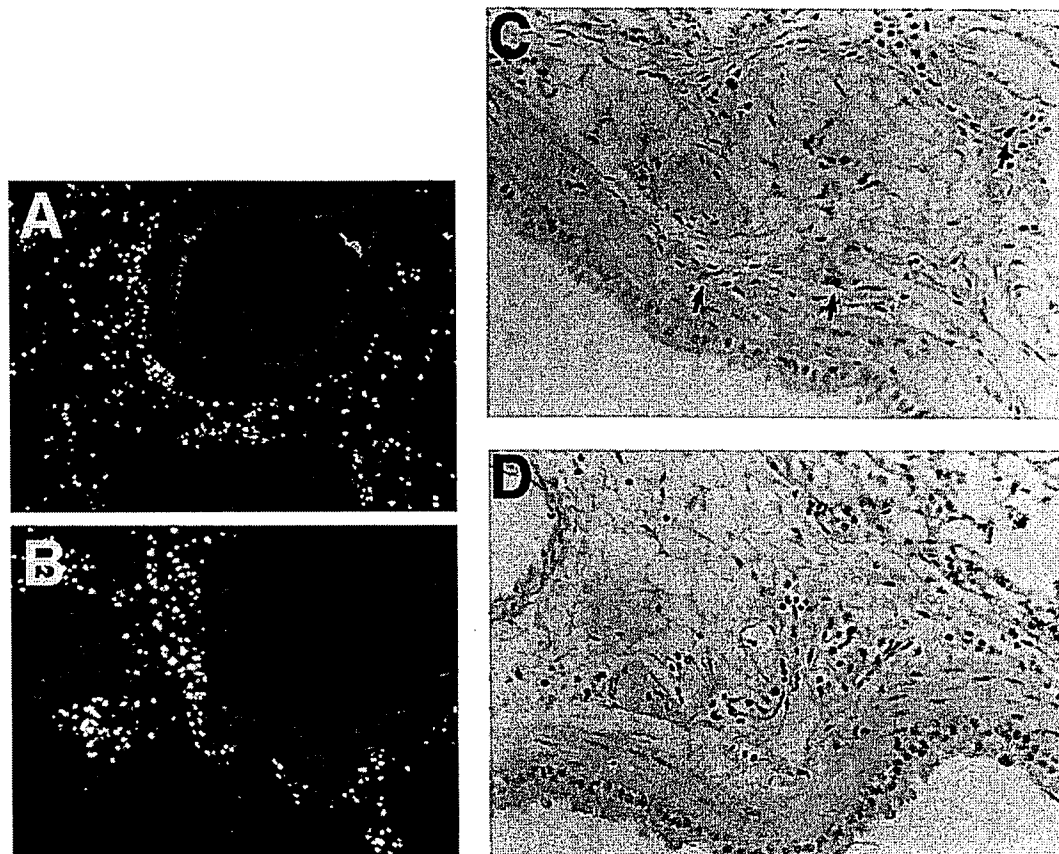


Figure 3. Immunohistochemistry of peribronchial tissue after sensitization and challenge with OVA. Localization of eosinophils and mast cells are shown. $+/+$ mice are shown in *a* and *c* and W/W^v mice in *b* and *d*. In *c* and *d*, cells were stained with Astra Blue/Vital New Red. For *a* and *b*, staining was with a rabbit anti-mouse MBP antibody and fluorescein-labeled goat anti-rabbit IgG. Original magnification of 500.

the congenic littermates after sensitization and challenge with OVA. Challenge alone on three occasions was insufficient to trigger antibody responses in either group of mice.

Eosinophilic Accumulation in the BAL and Lung. As shown in Fig. 2 *a*, sensitization and challenge with OVA had a marked effect on the numbers and composition of the cells recovered. In both groups of mice, macrophages were the predominant cell type in the mice receiving three challenges with OVA alone (similar to control mice; data not shown). However, after both sensitization and challenge, cell numbers increased and the predominant cells in the BAL were eosinophils, comprising roughly 60% of the cells in both the mast cell-deficient and congenic littermates.

When lung digests were examined, sensitization and challenge also resulted in a marked increase in eosinophil numbers (Fig. 2 *b*), although total cell numbers were little changed when compared with challenge alone. As in the BAL, there were no differences in the numbers of eosinophils in the lung digests between mice that were mast cell deficient or sufficient.

Localization of Eosinophils and Mast Cells in Lung Tissue. After staining with anti-MBP antibody, sensitization and

challenge significantly increased the numbers of eosinophils per area in the peribronchial tissue of both groups of mice (Fig. 3, *a* and *b*) to $187 \pm 23/\text{mm}^2$ in $+/+$ mice, $168 \pm 18/\text{mm}^2$ in W/W^v mice ($n = 4$). In animals challenged alone very few eosinophils were detected in these sites ($13 \pm 4/\text{mm}^2$). Staining with Astra Blue/Vital New Red revealed the accumulation of mast cells in the submucosal tissue of the bronchi in sensitized and challenged $+/+$ mice (Fig. 3 *c*). None could be identified in any of the sections examined from W/W^v mice (Fig. 3 *d*).

Airway Responsiveness. We examined baseline lung function and assessed the airway response to inhaled methacholine. Baseline (before MCh challenge) measures of lung function as assessed with RL and Cdyn are presented in Table 1. The values in all four groups were comparable. The response to aerosolized methacholine in mice that were challenged with antigen alone revealed small, dose-dependent changes in RL and a 20–30% dose-dependent fall in Cdyn (Fig. 4). After sensitization and challenge, resistance values increased by almost fivefold and dynamic compliance was reduced by 60–70% with comparable doses of methacholine. The responses in the mast cell-deficient mice, if any-

Table 1. Baseline Values of RL and Cdyn in Mice

Mice	Group	RL	Cdyn
		<i>cmH₂O</i> · <i>ml</i> ⁻¹ · <i>sec</i>	<i>ml</i> · <i>cm H₂O</i> ⁻¹
+/+	N	0.45 ± 0.057	0.039 ± 0.0012
W/W ^v	N	0.42 ± 0.070	0.039 ± 0.0013
+/+	ipNeb	0.40 ± 0.062	0.036 ± 0.0020
W/W ^v	ipNeb	0.44 ± 0.063	0.035 ± 0.0018

Lung resistance and dynamic compliance values in sensitized and challenged mice. RL and Cdyn values were obtained in the different groups of animals after sensitization and challenge but before exposure to MCh. The results for each group are expressed as means ± SEM (*n* = 8).

thing, exceeded the response in the congenic littermates, with a shift to the left in the methacholine dose-response curve for both RL and Cdyn.

Discussion

Mast cells and their released products are widely believed to contribute to the development of allergic respiratory disorders. IgE-dependent activation of mast cells can induce these cells to release a panel of preformed or newly synthesized mediators including histamine, tryptase, prostaglandins, leukotrienes, and platelet activating factor, which can result in acute phase allergic reactions in the lung including airway obstruction, airway microvascular leakage, and mucosal edema, as well as mucus gland hypersecretion (13–15). Although a role for mast cells has been defined in the acute phase of allergic reactions, much less is known about their role in chronic allergic lung inflammatory responses and their contribution to lung dysfunction in this setting. After allergen sensitization and challenge in the mouse, the changes in airway function that have generally been monitored include the response to MCh (8) or electrical field stimulation of tracheal smooth muscle preparations (16) and likely reflect a more chronic, eosinophil-dependent response (12). The current study extends previous investigations by assessing airway responsiveness *in vivo* and factors, such as cells and antibodies, which may contribute to the development of airway responsiveness.

Sensitization and challenge of the mast cell-deficient mice resulted in IgE and IgG1-specific antibody responses, increased eosinophils in the BAL and lung digests, and peribronchial infiltration of eosinophils. In all of these aspects, they were indistinguishable from their congenic littermates. The only difference was that mast cells were identified histologically in the submucosa of +/+ mice and not in the W/W^v animals. These findings suggest that the development of an allergic inflammatory reaction is not dependent on the presence of functional mast cells. These results are similar to what has been suggested in other systems. For example, Nogami and coworkers (17) showed no evidence for the involvement of mast cells in the pulmonary eosino-

RL and Cdyn changes in mice

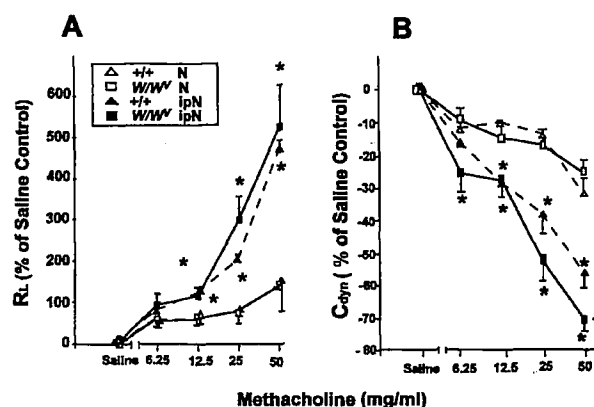


Figure 4. Lung resistance (A), and pulmonary dynamic compliance (B) in sensitized and challenged mice. RL and Cdyn values were obtained in response to increasing concentrations of methacholine as described in Materials and Methods. The results for each group are expressed as means ± SEM (*n* = 8). *Significant differences (*P* < 0.05) between the groups. *Significant differences (*P* < 0.05) between W/W^v and +/+ mice.

philic response to challenge with an extract from the parasite *Ascaris suum*. Further, Brusselle et al. (18) demonstrated no effect of mast cells on eosinophil influx in BAL fluid after repeated challenge with OVA in sensitized mice. In contrast, Kung et al. (19) reported that OVA challenge of sensitized mast cell-deficient mice produced fewer eosinophils in the BAL fluid and lungs compared with similarly sensitized and challenged congenic littermates. However, in this study both the sensitization and challenge protocol were attenuated and the number of eosinophils was significantly lower than we and others (18) generally observe after sensitization and challenge as described in this study. In their protocol, mice were challenged with antigen on only 1 d; in our studies we have found that 1 or 2 d of antigen challenge were not sufficient to develop airway hyperresponsiveness (our unpublished data). In our protocol, airway inflammation and eosinophil accumulation may have been sufficiently strong so that a role for the mast cells could not be demonstrated.

The presence of a comparable peribronchial eosinophil response in the W/W^v and +/+ mice was associated with a similar response to MCh in this study. Monitoring both resistance and dynamic compliance, aerosolized MCh resulted in a dose-dependent increase in RL as well as a 60–70% decrease in compliance. These changes were only observed in sensitized and challenged animals. At virtually all concentrations of MCh, the findings in the W/W^v mice exceeded those in the +/+ mice. At present, there is no apparent explanation for these differences. One possibility is that in the airways of mast cell-sufficient animals, heparin is released after activation (20), and may limit the response to the cationic protein mediators released by these same or other cells. In this regard, it has been previously shown that heparin sulfate and other polyanionic molecules block the

increase in airway responsiveness caused by highly charged cationic proteins (21, 22).

If sensitization and repeated challenge with antigen triggers both eosinophilic inflammation and AHR, does this eliminate a role for mast cells in the development of these changes? It is possible that the same physiologic response, AHR to MCh challenge, may be mediated by distinct cellular mechanisms in different strains of mice (23). For example, in BALB/c mice AHR could be induced in an IgE and mast cell-dependent fashion. In strains genetically deficient in important mast cell mediators (e.g., mast cell protease 7 deficiency in C57BL/6 mice) AHR may be more dependent on other cell types, such as eosinophils. Mast cell deficient mice of the same background as studied here do demonstrate reduced severity of anaphylaxis induced by anti-IgE treatment as well as reduced airway responsiveness to MCh shortly after systemic administration of anti-IgE (25, 26).

After limited bronchoprovocation mast cells may play a role in the liberation of cytokines such as IL-4, IL-5, and TNF- α (15); because of preformed stores, mast cells could provide the initial source of TNF- α in IgE-dependent reactions (27). However, we have shown in nude mice that were passively sensitized with IgE, that despite adequate mast cell degranulation, insufficient cytokines are liberated to trigger an eosinophilic response (28). A similar inconsistency centers around the role of IgE in triggering allergic

inflammation and AHR. Sensitization exclusively via the airways, which results in limited eosinophilic infiltration of the peribronchial regions, results in AHR that appears to be IgE dependent (29). On the other hand, after sensitization and challenge as performed in this study, AHR may be IgE independent but eosinophil dependent (our unpublished data). Similar results have recently been reported by Korsgren et al. (30) demonstrating normal development of eosinophilic airway inflammation in B cell-deficient mice. However, we can not discount that in the mast cell-deficient mice, the presence of a normal IgE response serves to trigger other cells expressing either high affinity IgE receptors, e.g., basophils, macrophages, or other cells expressing low affinity receptors (6) to release important proinflammatory mediators.

In summary, in this study of mast cell-deficient mice we have shown that after sensitization and airway challenge they are capable of developing an allergic antibody response and changes in airway resistance and dynamic compliance that are similar to their congenic littermates. Although this does not exclude contributions of mast cells in other aspects of chronic allergic inflammatory responses, it indicates that mast cells do not have an essential role in development of eosinophilic airway inflammation and airway hyperresponsiveness to MCh in mice sensitized and challenged as described in this report.

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EXHIBIT D

Review

Open Access

Measuring the lung function in the mouse: the challenge of size

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Abstract

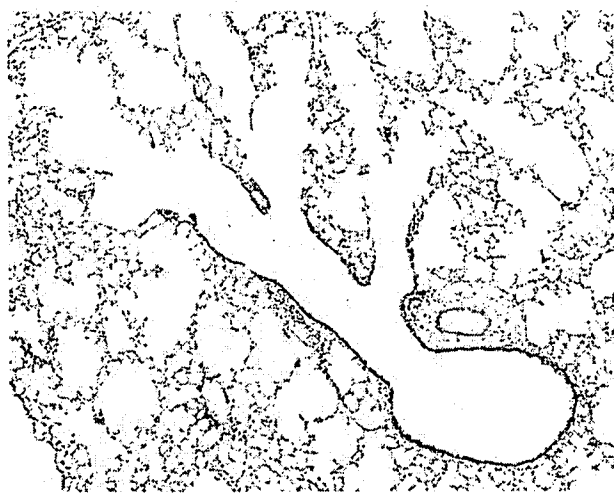
Measurement of the effects of drugs, mediators and infectious agents on various models of lung disease, as well as assessment of lung function in the intact mouse has the potential for significantly advancing our knowledge of lung disease. However, the small size of the mouse presents significant challenges for the assessment of lung function. Because of compromises made between precision and noninvasiveness, data obtained may have an uncertain bearing on the mechanical response of the lung. Nevertheless, considerable recent progress has been made in developing valid and useful measures of mouse lung function. These advances, resulting in our current ability to measure sophisticated indices of lung function in laboratory animals, are likely to lead to important insights into the mechanisms of lung disease.

Introduction

Much of our current understanding of the normal functioning of the lung and mechanisms of lung disease comes from studies utilizing animals. As one clear example, animal systems of a wide variety of species, including humans, provided the essential mechanistic proof of a link between inflammation and airways hyperresponsiveness that set the stage for current anti-inflammatory therapy [1]. Mice are now widely employed in lung research because of certain advantages this species is thought to provide [2]. Advantages of using mice include a well-understood immunologic system, the vast array of available reagents, a short reproductive cycle, a well-characterized genome, the advent of transgenic technology, and economic factors [2-4]. Using mice as models of human disease, in particular asthma, has certain shortcomings [2,5] only some of which will be covered in this review. For any animal system to yield useful and valid insights into disease it must exhibit an appropriate phenotype. It has be-

come apparent that the valid assessment of lung function in an animal as small as the mouse requires that a number of technical challenges be overcome.

The paucity of information on the measurement of lung function in the mouse has largely reflected the difficulty of measuring the necessary respiratory signals of flow, volume and transpulmonary pressure. This applies particularly to the small gas flows involved [6,7]. However, the work of Martin *et al* in 1988 demonstrated that measurements of pulmonary resistance and compliance could be made in this small species [8]. At about the same time, Levitt and Mitzner clearly illustrated the utility of using mice to explore the genetics of hyperresponsiveness [9,10]. Since these studies, the use of mice to study lung disease has increased dramatically and a number of approaches have been developed in the ensuing years for measuring lung function in mice *in vivo*. In this review we examine these various methods and discuss their

**Figure 1**

Photomicrograph of the parenchyma and respiratory bronchioles of a mouse (20 gram female BALB/c) lung. Note the rapid branching from a conducting airway into alveolar ducts and the relatively large airways. Stain is H & E with 5 × magnification.

respective attributes. Each approach represents a compromise between accuracy, non-invasiveness, and convenience [11].

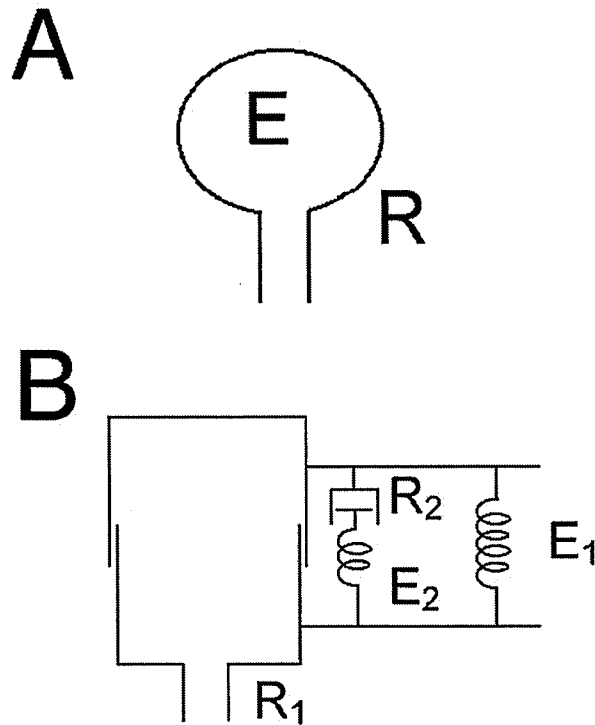
Lung anatomy

One look through the microscope at a section of mouse lung (Figure 1) demonstrates that the mouse lung is considerably different in structure from the human lung, although relatively little has been published about the architecture of the mouse lung compared to other species. What is known about the structure of the mouse lung probably has important bearing on its function [12–14]. The total lung capacity (TLC) of the mouse is about 1 ml compared to 10 ml of the rat and 6,000 ml of a human. Like the human, there are 5 lobes in the right mouse lung, but unlike the human the mouse has only a single left lung. Also unlike the human lung, but similar to the rat, the mouse pleura is thin, yet it is strong enough to be inflated to considerably higher pressures than the 30 cm H₂O normally associated with TLC (W Mitzner, personnel communications). The parenchyma of the mouse lung occupies a smaller fraction of the total lung than that of the rat but more than that of the human (mouse: 18%, rat: 24%, human: 12% lung volume). The alveoli of the mouse lung are smaller (80 μm mean linear intercept (MLI)) than those of the rat (MLI 100 μm) or human (MLI 210 μm). The blood-gas barrier thickness in the mouse (0.32 μm) is similar to that of the rat (0.38 μm) but some-

what smaller than that of the human (0.62 μm), which might have important implications for both gas exchange and parenchymal lung mechanics. The airways constitute a large percentage of the lung in mice (11%) compared to rats (5.7%). Cartilage is present in the mouse trachea but is less well organized than in other species; only the upper part of the trachea has the complete rings seen in other mammals and these rapidly change to plates as one proceeds distally. Mouse lungs have fewer respiratory bronchioles and airway generations (13–17 generations) than do human lungs (17–21 generations) with the airways of the mouse lung exhibiting a monopodial as opposed to dichotomous branching pattern. Two other significant features of the mouse lung are the thinness of the respiratory epithelium and the relatively large airway lumen [12,14]. This large airway caliber is speculated to reduce the flow-resistive load that would otherwise result from the rapid respiratory rate (250–350 bpm) required by the mouse to maintain body temperature [15]. An important functional difference between mice and rats compared to humans is the paucity, or even complete absence, of submucosal glands and the presence of high numbers of Clara cells [12]. Exactly what significance all these anatomical features of the mouse lung have for lung function is speculative, but it has been our experience that the baseline airway resistance of mice that have been sensitized and challenged with antigen differs imperceptibly from that of control animals [16,17]. This suggests that inflammatory processes that could compromise lung function in larger animals (e.g. humans) might have little effect in mice because of their relatively large airway size and/or lack of mucous glands.

Basic mechanical models of the lung

Measurement of the function of the lung, especially assessment of lung mechanics, is typically done in the context of a model of the lung [18–20]. The simplest model is a tube connected to a bellows (Figure 2A). This model works well for a single breathing frequency, but has major limitations when the changes in lung mechanics that occur with alterations in breathing frequency are considered. This is because the resistive and elastic properties of the lung are substantially dependent on breathing frequency. For example, the resistance of the lung falls as frequency increases over the range associated with normal breathing [21]. To model this type of mechanical behavior, spring-and-dashpot assemblies capable of simulating viscoelastic behavior need to be included in the model (Figure 2B). These basic models allow us to develop mathematical expressions, which can be used to quantitatively assess lung mechanics. The parameters of the models, that is, the resistive and elastic values of their individual components, constitute the endpoints we use to assess lung function experimentally.

**Figure 2**

Two common and basic mechanical models of the lung. A: A homogeneously ventilated model consisting of a single elastic balloon (elastance E) served by a single flow-resistive pipe (resistance R). B: A homogeneous model again with a single airway (resistance R_1), but with a Kelvin body consisting of two springs (E_1 and E_2) and a dashpot (resistance R_2) to account for the viscoelastic behavior of the lung tissue.

The viscoelastic model in Fig. 2B does a substantially better job of describing frequency-dependent nature lung mechanics than the model in Fig. 2A. Nevertheless, the simple model in Fig. 2A still serves as the conceptual platform for most studies of lung mechanics and bronchial responsiveness. The mechanical behavior of this model is described by its equation of motion. This equation is based on simple physics and states that the force (pressure) applied to the model is equal and opposite to the opposing force (pressure) the model generates. The applied pressure, P , is that supplied either by the respiratory muscles or a mechanical ventilator. The opposing pressure is made up, in general, of three components: a resistive pressure (P_{res}), an elastic pressure (P_{el}), and an inertive pressure (P_{in}).

Thus,

$$P = P_{res} + P_{el} + P_{in} \quad (\text{Equation 1})$$

P_{res} is described by Ohm's law:

$$P_{res} = R\dot{V} \quad (\text{Equation 2})$$

where R is the resistance of the lung and \dot{V} is flow of gas. P_{el} is described by Hooke's law:

$$P_{el} = EV = \frac{1}{C}V \quad (\text{Equation 3})$$

where E is lung elastance (equal to the inverse of compliance, C) and V is lung volume relative to functional residual capacity. P_{in} comes into play only at frequencies well above those of normal breathing, while both P_{res} and P_{in} become negligible when frequency is extremely low. Thus, the equation of motion relevant to normal breathing is

$$P = R\dot{V} + \frac{1}{C}V \quad (\text{Equation 4})$$

The parameters R and E are both profoundly dependent on breathing frequency and lung volume.

Lung volume

The volume of the lungs has an important influence on its pressure-flow relationships. For example, an increase in lung volume stretches the airways open and so causes airway resistance to fall (tethering). This also makes it more difficult for the airways to narrow when the airway smooth muscle contracts, and represents an important mechanism by which the challenged lung can defend airway caliber [18,22]. Unlike larger laboratory animals, the measurement of lung volume in the mouse is particularly problematic due to its small size. For example, when thoracic gas volume is measured using the conventional Boyle's Law technique, the volumes of air in the transducers used to measure plethysmographic and airway-opening pressures must be small relative to the lung volume, or significant measurement errors will occur. It has only recently been reported that measurement of functional residual capacity (FRC) by this approach is at all feasible [23]. The measurement of FRC with gas dilution is equally difficult, again due to the small size of the mouse lung, and there are only a few reports in the literature on the use of this technique [24]. Other studies of mouse lung volume have used a buoyancy approach [25], a degassing approach [26,27], and even a CT scanner method has been reported [28]. None of these, however, is particularly practical for most study designs. Better techniques for measuring lung volumes in mice are certainly needed, so this will be a fruitful area for future research.

Lung elastance (compliance)

The component of the transpulmonary pressure loss that is out of phase with flow and in phase with volume, as well as the recoil pressure exerted by the lung under static conditions, are caused by the elastic forces within the lung. The loss of elastic recoil within the lung defines emphysema while an increase defines restrictive processes [18,25]. The chest walls and other thoracic structures in mice are extremely compliant, so most elastic recoil measured in an intact animal can be attributed specifically to the lung. Moreover, the elastic recoil of the lung shows considerable genetic variability that needs to be taken into account in study designs [26]. The elastic recoil of the lung is conveniently assessed in terms of the quasi-static pressure-volume (PV) curve measured by inflating and deflating the lung in a step-wise fashion. The inspiratory limb of the curve traverses a path through values of P that are higher than those of the expiratory limb, the difference between the two limbs being termed hysteresis. Changes in the inspiratory limb of the PV curve that cause an increase in hysteresis are taken to indicate enhanced airway closure, such as that observed in humans after dry cold gas inhalation [29] and recapitulated in mice with allergic inflammation [30]. These changes in PV characteristics can be sensitive indicators of lung dysfunction and contribute to the genesis of hyperresponsiveness. The shape of the pressure volume relationship is one manifestation of the nonlinear characteristics of lung mechanics in the normal, unperturbed lung. Airflow resistance also exhibits alinear behavior as the airflow reaches high rates of flow as sudden changes in luminal dimensions occur (e.g. vocal chords). The mouse lung exhibits alinear elastic (compliance) behavior that increases following antigen challenge, a change that is most consistent with reopening airways that were closed [29,30]. Airflow is not alinear (i.e. laminar flow regimes) in either condition as it is highly unlikely turbulent flow occurs in mouse lungs due to the small airway diameters, unlike humans where turbulent flow is a common occurrence [30], pointing to a clear limitation of this species in exploring complex airflow conditions.

Phenotyping uncertainty principle

Accurate and valid measurement of lung mechanics in laboratory animals is a balancing act between measurement precision and maintenance of "natural" conditions. This situation is similar to the Heidelberg uncertainty principle of quantum mechanics which states that the measurement of a particle's position interferes with the measurement of its movement, and *vice versa* [31]. In a similar fashion, as we make more precise measurements of lung function in an animal, we are forced to constrain the animal's behavior in a way that departs from the maintenance of natural conditions [11]. At the extreme ends of this continuum are the measurements derived from the free roaming animal in a closed chamber, known

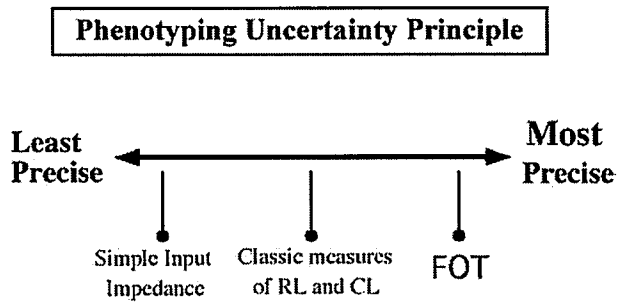


Figure 3

The non-invasiveness-precision continuum of the phenotyping uncertainty principle – see text for discussion.

as unrestrained plethysmography (UP), and the measurement of input impedance using forced oscillations performed in an anesthetized, tracheostomized animal (Figure 3).

Unrestrained plethysmography

This approach to assess lung function involves placing the subject into a small closed box and measuring the pressure changes within the box that occur as the animal breathes [7,11,32]. The animal is conscious and unrestrained. This technique currently enjoys wide popularity (for example see [33]) because 1) it is simple and 2) the mouse remains unharmed after the experiment. The endpoint is the heuristic variable known as Penh, which stands for 'enhanced pause'. It is important to note that there is no linkage between Penh and other variables that are derived from mechanical principles – Penh is merely an empirical derivative of the respiratory variations in box pressure [11]. While an earlier publication demonstrated reasonable correlations between Penh and invasive measures of lung mechanics [32], recent publications draw into serious question the validity of using Penh to measure lung function [7,11,34].

The pressure changes occurring within the box as the mouse breathes are derived first from gas compression and decompression within the thorax – an event linked to the state of lung mechanics – and second from humidification and warming of inspired gas – an event unrelated to lung mechanics. During bronchoconstriction, both components increase [7], but much of this increase is likely due to the increased stimulation to breathe that would arise from chemoreceptor receptors in the lung. Hence box pressure changes should be influenced by chemoreceptor sensitivity and genetics that control responses to chemo- or irritant- receptor stimulation and integration [11,35]. Recent studies show that

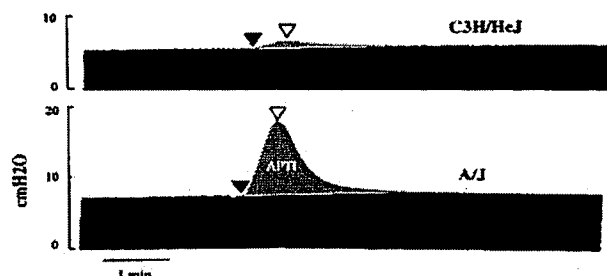


Figure 4

Pulmonary impedance measurements in anesthetized mice. The diagram shows airway opening pressure plotted against time. Volume and flow excursions for each breath are maintained constant by use of a volume-cycled ventilator. Increases in the magnitude of lung impedance following acetylcholine injection are assessed as the increase in pressure above baseline. Note the increased responsiveness in the A/J compared the C3He/J strain of mouse. APTI: Airway pressure time index. Used with permission [38].

changes in $Penh$ depart from mechanical changes during a state of increased box temperature [7,34] in an exactly opposite way during exposure to hyperoxic conditions [34,35] and temporarily [36]. These findings show that $Penh$ is not a valid measurement of the lung function of the mouse except as a measure of patterns of respiration, and it has been known for a long time that patterns of respiration usually have little bearing on lung mechanics. Finally, a response in $Penh$ may also be due to changes in nasal cavity resistance, as the upper airways are very significant contributors (50%) to total lung resistance and their contribution is likely to change depending on the experimental situation [32].

Lung impedance magnitude

The next step on the phenotyping uncertainty continuum (Figure 3) is the measurement of the magnitude of respiratory system or lung impedance. Lung impedance is a complex quantity having both real and imaginary parts (see section 'Forced oscillations and the constant phase model'), and its calculation requires rather sophisticated methods. The magnitude of impedance ($|Z_{rs}|$), however, is easily determined simply as the ratio of the absolute value of the swing in pressure (ΔP) to the absolute value of the swing in flow ($\Delta \dot{V}$) occurring over a breath, thus

$$|Z_{rs}| = \frac{|\Delta P|}{|\Delta \dot{V}|} \quad (\text{Equation 5})$$

As seen in the example in Figure 4, pressure is obtained by placing a pressure transducer at the airway opening, while flow is assumed to be constant as the animal is mechanically ventilated with a volume-cycled ventilator [9,10,37,38]. When a bronchoactive agent is introduced, the peak pressure with each breath goes up, so $|Z_{rs}|$ increases commensurately. Hence, by merely measuring airway-opening pressure, a useful index of lung function is derived. This technique has been used because it is simple and gives a direct assessment of lung mechanics [9,10,37,38].

The major disadvantage of this technique is that even though a direct measure of lung function is made, no insight is obtained as to where in the lung an abnormality might be located. This is a significant limitation if one wishes to explore the mechanisms of bronchoconstriction and whether it reflects, for example, central versus peripheral airways dysfunction. Nevertheless, this simple approach has produced significant advances in our understanding of the genetics of hyperresponsiveness [3,9,10].

Measurement of dynamic resistance (R_L) and compliance (C_L)

A classic approach to assessment of lung mechanics in animals is the measurement of dynamic lung resistance (R_L) and compliance (C_{dyn} or C_L) [3,8,20,38–40]. In the past, this approach was often used to assess central versus peripheral alterations in lung mechanics – a topic of considerable current interest. The calculation of R_L and C_{dyn} requires the measurement of intrathoracic pressure that, in larger animals, is obtained with an esophageal balloon or pleural catheter, but in a mouse is obtained either by opening the chest or by making the reasonable assumption that the chest wall presents little mechanical load compared to that of the lung [26,41,42]. Flow is usually obtained with a pressure transducer but this approach is problematic when miniaturized to the mouse [7,43]. Accordingly, flow is commonly derived from the differentiation of a volume signal, usually obtained from a body plethysmograph [8,40]. The values of R_L and C_L are then derived by fitting the equation of motion (Equation 4) to measurements of pressure, flow and volume.

The measurement of R_L and C_L , while technically challenging, does yield additional insight into the mechanisms of bronchoconstriction over that provided by $|Z_{rs}|$. Generally speaking, an increase in R_L reflects both narrowing of the conducting airways and alterations in the lung periphery (heterogeneous narrowing or closure of distal airways together with changes in the intrinsic mechanical properties of the parenchyma). Decreases in C_L , on the other hand, reflect only events in the lung periphery, particularly airway closure leading to lung unit derecruitment [44]. If the response to an intervention is limited largely

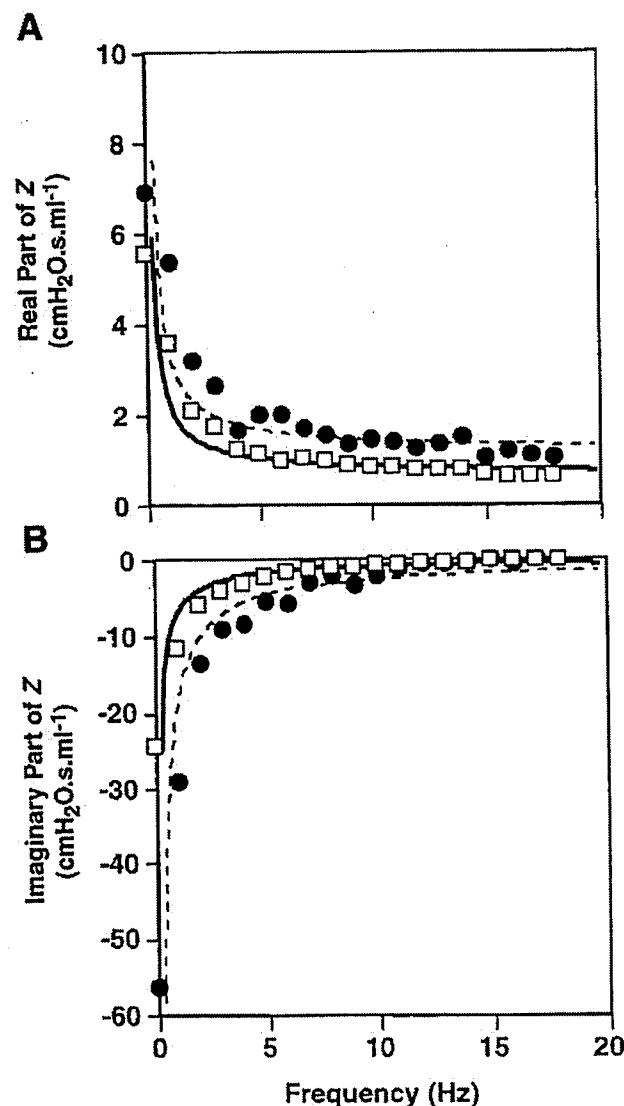


Figure 5

The respiratory input impedance of the mouse. Open squares represent baseline conditions while closed circles show the result of administering an aerosol of methacholine. The solid and dashed lines are the fit provided by the constant-phase model (Eq. 6). Used with permission [17].

to R_L , then a relatively proximal location is implicated for the effect. By contrast, a selective change in C_L is indicative of a more distal site of action [3,8,45]. As an example of this approach, R_L and C_L were clearly shown to be independent variables in mice treated with an antibody agonist for VLA-4, an adhesion protein of the eosinophil [45]. Furthermore, the genetic dependence of these variables

suggests that the factors that control central airway function (reflected in R_L) are different from those that control peripheral airway function (reflected in C_L) [46].

Forced oscillations and the constant phase model

At the far end of the phenotyping uncertainty principle lies the forced oscillation technique (FOT) applied in anesthetized, paralyzed, tracheostomized animals to measure the complex input impedance (Z_{rs}) of the lungs [21]. We have already covered the concept that the magnitude of Z_{rs} ($|Z_{rs}|$) is a generalization of the changes of resistance and compliance, and that Z_{rs} consists of two parts that are both functions of frequency. The real part of Z_{rs} is directly related to the resistance and provides essentially the resistance of the respiratory system at the frequency in question. The imaginary part of Z_{rs} is called the reactance and reflects respiratory compliance at frequencies below 20 Hz in the mouse. Thus, the FOT essentially provides R_L and C_L at each frequency contained in the flow signal applied to the lungs. This requires that the animal be oscillated with a complex flow wave produced by either a loudspeaker [34,47,48] or a computer-controlled piston [6,7,17]. The data of pressure and either flow or volume are converted into the frequency domain by the Fast Fourier transform, and their ratios calculated to yield the real and imaginary parts of Z_{rs} (Figure 5).

The key advantage of this approach, as compared to the determination of R_L and C_L or $|Z_{rs}|$, is that Z_{rs} can be fitted to a more complex model of the lung known as the constant-phase model [49] which makes a clearer distinction between central and peripheral events in the lung. The equation of motion of the constant-phase model is

$$Z_{in}(f) = R_{aw} + i2\pi f I_{aw} + \frac{G_{ti} - iH_{ti}}{(2\pi f)^\alpha} \quad (6)$$

where R_{aw} is the resistance of the airways that are attached to the constant phase element, I_{aw} is the inertance of the gas in the airways (which has negligible effect in the mouse below 20 Hz and can be ignored [17]), G_{ti} is tissue resistance or damping, H_{ti} is tissue elasticity, and i is $\sqrt{-1}$. As R_{aw} is a measure of central airways resistance, it would be expected to change if the airways are significantly narrowed. By contrast, G_{ti} reflects either changes in tissue physical properties or regional airways heterogeneity. If changes in R_{aw} are small, then any changes in G_{ti} most likely represent changes in the parenchyma or very small airways. Acute changes in H_{ti} are likely to reflect lung derecruitment (airway closure) [44], whereas chronic changes in H_{ti} would be expected to reflect changes in the intrinsic mechanical properties of the parenchyma. This technique

is now being successfully and extensively used to assess lung mechanics of the mouse [17,33,50].

Tomioka et al [17] showed that Z_{rs} can be used to track changes obtained with an even more invasive technique [51,52] – the alveolar capsule – where resistance is partitioned into a central airway and a parenchymal tissue resistance component through the direct measurement of alveolar pressure (Figure 6). In this study, the disparate behaviours of R_{aw} , G_{ti} and H_{ti} clearly show that these three quantities are independent parameters that access different aspects of lung function. For example, antigen exposure followed by methacholine challenge caused an enhancement of both G_{ti} and H_{ti} – measures of peripheral lung function – however, changes in R_{aw} , reflecting central airways, were not significantly altered. The enhanced changes in G or H in this acute state may reflect three different mechanisms: derecruitment of lung units as airways close, temporal shifts of tissue movement, and inhomogeneities of airflow distribution. Moreover these data point away from significant alterations in airway smooth muscle function and more towards enhanced or altered secretions that cause dysfunction in small airways. Interestingly, antigen challenge in either sensitized or unimmunized mice caused no significant changes in any of the parameters at baseline before methacholine challenge, likely due to the unique architecture of the mouse lung (see above).

We believe that the well-founded theoretical basis of the FOT, and its rigorous application in mice, will lead to considerable insight into the functioning of mouse models of lung disease.

Conclusion

Measurement of lung function in a creature as small as the mouse presents considerable technical challenges. However, with the exception of the measurement of absolute lung volume and the analysis of blood gases, we have now conquered the challenge of miniaturizing the instrumentation necessary for mouse lung function assessment. Application of advanced techniques such as the FOT coupled with constant-phase model analysis hold particular promise for improved characterization of lung responses to intervention and pathology. With these approaches, we can now unravel the mechanisms of airways dysfunction, the influence of genetics and the immunological factors that define the physiome of the mouse.

Abbreviations

bpm breaths per minute

C_{dyn} dynamic compliance

C_L lung compliance

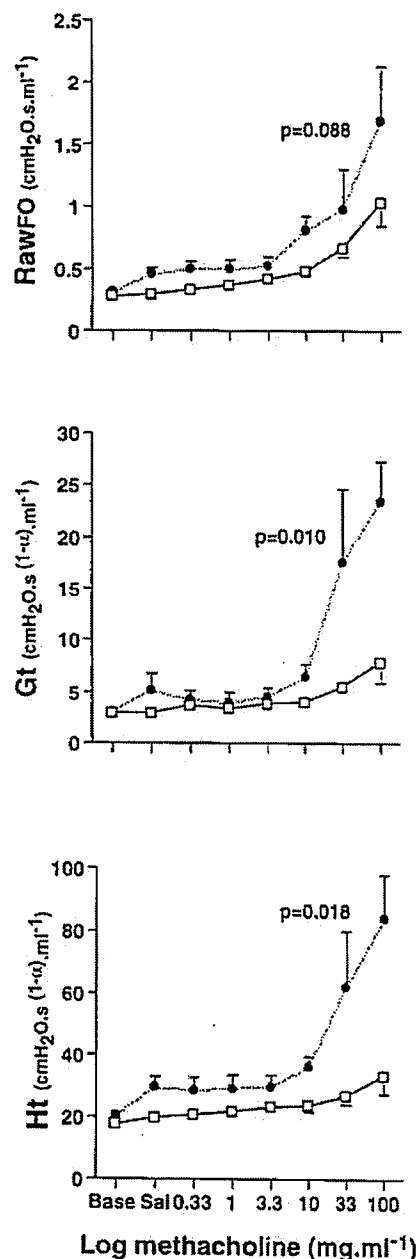


Figure 6

The mechanical response of the mouse lung to methacholine in terms of the parameters of the constant-phase model (see Eq. 6 in text). The open squares correspond to control BALB/c mice, while the closed circles represent mice sensitized to and challenged with ovalbumin. Taken from [17] with permission.

E elastance

FOT forced oscillation technique

FRC functional residual capacity

G tissue damping or tissue resistance

H elasticity

MLI mean linear intercept

P pressure

P_{res} resistive pressure

P_{el} elastic pressure

P_{in} inertial pressure

Penh enhanced pause

R_L resistance

TLC total lung capacity

UP unrestrained plethysmography

\dot{V} Flow

Z_{RS} or Z impedance of the respiratory system

$|Z_{rs}|$ Magnitude of impedance

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APPENDIX H

Constructive reduction to practice the proposed count in U.S. Provisional Application No. 60/189,622

Proposed Count	Representative Support in U.S. Provisional Application No. 60/189,622 (the '622 application)
<p>A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);</p> <p>wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.</p>	<p>For the experiment described in the '622 application, page 5, lines 16 to 24, discusses the sensitization and challenge of the mice with ovalbumin (OVA), as well as the negative control mice that were not both sensitized and challenged with OVA. As discussed in detail in the Dakhama and Kanehiro Declarations, sensitization and challenge with OVA according to this experiment creates a mouse that has allergen-induced airway hyperresponsiveness. See Dakhama and Kanehiro Declarations at paragraphs 8 to 10.</p> <p>The '622 application at page 5, line 25, to page 6, line 5, discusses the assessment of airway function for the experiment. "Airway function was assessed in vivo in anesthetized, mechanically ventilated mice as previously described (see, for example, Takeda et al., (1997). <i>J. Exp. Med.</i> 186, 449-454) by measuring changes in lung resistance (R_L) and dynamic compliance (C_{dyn}) in response to intra-tracheal challenge with aerosolized methacholine at doses of 1.56, 3.125, 6.25 and 12.5 mg/ml saline. Baseline values were recorded from data obtained after intra-tracheal challenge with aerosolized saline. The data are presented in percent of change from baseline R_L and C_{dyn}." As discussed in detail in the Dakhama and Kanehiro Declarations, exposing the mice to methacholine in increasing doses and measuring baseline R_L and C_{dyn} in this manner "was an acceptable method of testing whether a test agent inhibited allergen-induced AHR in mammals compared to mammals that did not receive the treatment with the test agent." See Dakhama Declaration at pages 5 to 7, paragraphs 11 to 15; and Kanehiro Declaration at pages 5 to 8, paragraphs 11 to 15. As discussed in those Declarations, "[i]ncreases in R_L and decreases in C_{dyn} correlate with increased AHR." See Dakhama Declaration at pages 6 to 7, paragraph 13; and See Kanehiro Declaration at page 7, paragraph 13.</p> <p>The '622 application at page 9, line 22, to page 10, line 11, and at Figures 4 and 6, provides the results of the experiments comparing mice with allergen-induced airway hyperresponsiveness that were administered CGRP (designated "OVA +CGRP" in those figures) to mice with allergen-induced airway hyperresponsiveness that were not administered</p>

Proposed Count	Representative Support in U.S. Provisional Application No. 60/189,622 (the '622 application)
	<p>CGRP(designated "OVA" in those figures). Figures 4 and 6 show a decrease in R_L and an increase in C_{dyn} for the mice administered CGRP as compared to the mice that were not administered CGRP. Thus, the experiment showed that administration of CGRP to mice with allergen-induced AHR inhibited allergen-induced AHR in those mice as compared to in the absence of administration of CGRP. These results are discussed in the '622 application at page 10, lines 1 to 11, and at page 11, lines 1 to 5.</p>

APPENDIX I

Constructive reduction to practice the proposed count in the '753 application

Proposed Count	Representative Support in the '753 application
<p>A method to inhibit allergen-induced airway hyperresponsiveness in a mammal, comprising administering to a mammal a calcitonin gene related peptide (CGRP);</p> <p>wherein said mammal has allergen-induced airway hyperresponsiveness, and wherein administration of said CGRP inhibits allergen-induced airway hyperresponsiveness in said mammal as compared to in the absence of administration of said CGRP.</p>	<p>For the experiment described in the '753 application, page 55, line 22, to page 56, line 2; page 60, lines 6 to 7; and page 61, lines 25 to 26, discuss the sensitization and challenge of the mice with ovalbumin (OVA), as well as the negative control mice that were not both sensitized and challenged with OVA. As discussed in detail in the Dakhama and Kanehiro Declarations, sensitization and challenge with OVA according to this experiment creates a mouse that has allergen-induced airway hyperresponsiveness. See Dakhama and Kanehiro Declarations at paragraphs 8 to 10.</p> <p>The '753 application at page 60, lines 6 to 13, and at page 61, lines 25 to 26, discusses the assessment of airway function for the experiment. "Airway function was assessed in vivo in anesthetized, mechanically ventilated mice as previously described (see, for example, Takeda et al., (1997). <i>J. Exp. Med.</i> 186, 449-454) by measuring changes in lung resistance (R_L) and dynamic compliance (C_{dyn}) in response to intra-tracheal challenge with aerosolized methacholine at doses of 1.56, 3.125, 6.25 and 12.5 mg/ml saline. Baseline values were recorded from data obtained after intra-tracheal challenge with aerosolized saline. The data are presented in percent of change from baseline R_L and C_{dyn}." As discussed in detail in the Dakhama and Kanehiro Declarations, exposing the mice to methacholine in increasing doses and measuring baseline R_L and C_{dyn} in this manner "was an acceptable method of testing whether a test agent inhibited allergen-induced AHR in mammals compared to mammals that did not receive the treatment with the test agent." See Dakhama Declaration at pages 5 to 7, paragraphs 11 to 15; and Kanehiro Declaration at pages 5 to 8, paragraphs 11 to 15. As discussed in those Declarations, "[i]ncreases in R_L and decreases in C_{dyn} correlate with increased AHR." See Dakhama Declaration at pages 6 to 7, paragraph 13; and See Kanehiro Declaration at page 7, paragraph 13.</p> <p>The '753 application at page 61, line 23, to page 62, line 9, and at Figures 3 and 5, provides the results of the experiments comparing mice with allergen-induced airway hyperresponsiveness that were</p>

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	<p>administered CGRP (designated "OVA +CGRP" in those figures) to mice with allergen-induced airway hyperresponsiveness that were not administered CGRP(designated "OVA" in those figures). Figures 3 and 5 show a decrease in R_L and an increase in C_{dyn} for the mice administered CGRP as compared to the mice that were not administered CGRP. Thus, the experiment showed that administration of CGRP to mice with allergen-induced AHR inhibited allergen-induced AHR in those mice as compared to in the absence of administration of CGRP. These results are discussed in the '753 application at page 61, line 27, to page 62, line 9.</p>